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The Rho GTPases Rac1, Cdc42, and RhoA Regulate APP Transport to Lysosomes and A β Production

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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The Rho GTPases Rac1, Cdc42, and RhoA Regulate APP Transport to Lysosomes
and A β Production

(Thesis format: Monograph)

by

Justin Chiu

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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Abstract

Alzheimer's Disease (AD) is characterized by Beta-Amyloid ($A\beta$) plaques within the brain. $A\beta$ peptides are produced by the cleavage of Amyloid Precursor Protein (APP). Our laboratory has previously discovered a novel pathway for APP internalization mediated by ADP-ribosylation factor 6 (Arf6). This pathway resembles macropinocytosis, transporting cell surface APP directly to lysosomes, a possible site for $A\beta$ production. We set out to characterize the effectors downstream of Arf6. In SN56 and N2A cells we co-transfected HA-tagged APP (to label cell-surface APP) with compartment markers, to visualize APP trafficking. We used dominant negative and constitutively active mutants, pharmacological inhibitors, and siRNA for Rac1, Cdc42, and RhoA to determine their roles in APP macropinocytosis. APP trafficking to lysosomes was reduced after knockdown of Rac1, Cdc42, and RhoA, and inhibition of this transport reduced production of $A\beta$ 40 and $A\beta$ 42. Our findings indicate a role for Rac1, Cdc42, and RhoA in $A\beta$ production.

Keywords

Alzheimer's Disease, APP, $A\beta$ 40, $A\beta$ 42, Arf6, Rac1, Cdc42, RhoA, macropinocytosis, lysosomes, intracellular trafficking, confocal microscopy

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List of Abbreviations

A β	Beta-Amyloid
AchE	Acetylcholinesterase
AD	Alzheimer's Disease
ADDL	Amyloid Derived Diffusible Ligands
AICD	APP intracellular domain
AP-2	Adapter protein-2
AP-3	Adapter protein-3
APLP1	Amyloid precursor-like protein 1
APLP2	Amyloid precursor-like protein 2
APP	Amyloid precursor protein
APPL	β amyloid protein precursor-like
Arf6	ADP-ribosylation factor 6
BACE	Beta-site APP-cleaving enzyme
bafA1	bafilomycin A1
CAV1	Caveolin-1
CAV2	Caveolin-2
CAV3	Caveolin-3
CHO	Chinese hamster ovary
CIE	Clathrin-independent endocytosis
CLIC/GEEC	clathrin-independent carriers/ GPI-AP enriched early endosomal compartments
CME	Clathrin-mediated endocytosis
CR1	Complement receptor-1
CTF	COOH-terminal fragment
dbcAMP	dibutyryl cyclic AMP
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EE	Early Endosome
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer's Disease
FBS	Fetal Bovine Serum
FE65L1	FE65-like 1
GEF	Guanine nucleotide exchange factor
GPI-Aps	Glycosyl phosphatidylinositol-anchored proteins
GTP	Guanosine triphosphate
HA	Hemagglutinin
HBSS	Hank's balanced salt solution
IDE	Insulin-degrading Enzyme
IgG	Immunoglobulin G
JNK	c-Jun amino terminal kinase

LAMP-1	Lysosomal-associated membrane protein-1
LTP	Long-term potentiation
M6PR	Mannose 6-phosphate receptor
MEM	Minimum essential media
N2A	Neural-2-A
NFT	Neurofibrillary tangle
NH ₄ CL	Ammonium Chloride
N-WASP	Neural Wiskott-Aldrich syndrome protein
PA	Phosphatidic Acid
PAK1	p21-activated kinase-1
PC	Phosphatidylcholine
PI3K	Phosphatidylinositol 3-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PLD	Phospholipase D
PrP ^C	Prion protein C
PVDF	Polyvinylidene fluoride
RER	Rough endoplasmic reticulum
ROCK	Rho-associated coiled-coil containing protein kinase
SNX5	Sorting nexin 5
TGN	Trans-Golgi Network
V-ATPase	Vacuolar type H ⁺ -ATPase
WAVE	WASP family Verprolin-homologus

Chapter 1: Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia. AD is a progressive, neurodegenerative disease uniquely characterized by the deposition of beta-amyloid ($A\beta$) plaques in the brain. AD's main risk factor is age, with incidence rate increasing substantially every 5 years starting at 65 years of age, with a 14 times higher occurrence compared to those 85 and older (Hebert *et al.* 1995). Current estimates by the Alzheimer's Society of Canada suggest that roughly 600,000 Canadians have dementia, and rising to over 1 million cases by 2038 (Smetanin *et al.* 2009). Global estimates either mirror or are higher than the predicted Canadian estimates, with some continental regions expecting a 5-times increased prevalence between 2006 to 2050 (Brookmeyer *et al.* 2007). With a quickly aging population, one of the greatest worries related to AD is the heavy economic burden that will be placed on the healthcare system. With an estimated cost of \$15 billion according to a 2008 estimate and increasing to \$160 billion by 2038 for Canada, AD will become one of the costliest and most challenging diseases in just a few decades (Smetanin *et al.* 2009).

1.2 Beta-Amyloid

Alzheimer's disease is characterized by synapse and neuronal loss, the appearance of neurofibrillary tangles, and senile plaques in the brain. Of these characteristics, only the development of senile plaques is unique to AD. Amyloid plaques are comprised mainly of aggregates of beta-amyloid (Walsh *et al.* 2007; Wenk 2003). As a result of the strong association between amyloid and Alzheimer's disease, the current leading hypothesis for the pathogenesis of Alzheimer's disease is the amyloid hypothesis.

1.2.1 Production of A β

A β peptides are produced from the sequential cleavage of the transmembrane protein, amyloid precursor protein (APP). First, APP is cleaved at an extracellular β -site by a β -secretase. In neurons, this initial β -cleavage is the preferred pathway for the first step of APP processing (Sinha *et al.* 1999). This secretase has been previously identified as an endosomal transmembrane aspartic protease named beta-site APP-cleaving enzyme (BACE) (Huse *et al.* 2000). The initial β -cleavage releases sAPP β , a roughly 100 kDa, N-terminal soluble fragment, leaving a membrane bound 12 kDa C-terminal fragment termed C99. Alternatively, APP can undergo α -cleavage instead through an α -secretase at the plasma membrane to instead produce sAPP α , another large ~100 kDa N-terminal soluble fragment, and C83, a 10 kDa C-terminal fragment (Thinakaran *et al.* 2008). Further cleavage of the C83 fragment leads to a shortened 3 kDa fragment (p3), containing only part of the A β sequence (Haass *et al.* 1993). As a result, α -cleavage of the APP protein produces a non-pathological peptide, and the α -cleavage pathway is non-amyloidogenic. In the amyloidogenic pathway, which generates the toxic peptide isomers, the C99 fragment remaining in the membrane is subsequently cleaved at a variable γ -cleavage site by the presenilin protein. Presenilin is a part of the γ -secretase complex, which has been shown to be active in a number of compartments such as the endoplasmic reticulum and endosomes (Kimberly *et al.* 2000, Thinakaran *et al.* 2008). This secondary cleavage of APP produces 36 to 43 amino acid long A β peptides and an APP intracellular domain (AICD). The A β peptides generated in the greatest quantities are A β 40 and A β 42. Of the two A β 42 is generated in significantly lower quantities; however, A β 42 is more toxic and much more prone to aggregation and thus generates amyloid plaques more easily (Iijima *et al.* 2008). These pathways are summarized in Figure 1.

1.2.2 Amyloid Precursor Protein

Human APP is part of a family of Type-1 transmembrane glycoproteins including amyloid precursor-like protein 1 (APLP1) and amyloid precursor-like protein 2 (APLP2), in addition, the APP gene is located on chromosome 21 (Nicolas *et al.* 2014; Hardy *et al.* 2002). While APLP1 and APLP2 show functional redundancy with APP, neither of the two has the A β sequence. APP is highly conserved, extending all the way down to invertebrates such as *Drosophila*, in which the orthologue is β amyloid protein precursor-like (APPL) (Rosen *et al.* 1989). These proteins all exhibit a large extracellular C-domain, and a short cytoplasmic N-domain. The APP gene itself can undergo alternative splicing producing multiple different APP isoforms. The major APP mRNA species are APP751, APP770, and APP695, with APP695 showing neuron-specific distribution and APP751 and APP770 being expressed ubiquitously (Coburger *et al.* 2014).

1.2.2.1 Physiological Function of APP

The evolutionary conservation of APP and its homologues signify an important role in normal physiology unrelated to the progression of Alzheimer's disease. In mouse embryos, APP was shown to increase in expression correlating with neuronal growth, and to potentially be a marker for developing nuclei in the brain (Salbaum *et al.* 1994). While some studies indicate it enhances neurite elongation, others suggest that it instead inhibits elongation (Nicolas *et al.* 2014). Similarly, in the mouse fetal and postnatal brain, APP was shown to be highly expressed in glial cells and neurons, especially during neuronal differentiation (Trapp *et al.* 1994; Nicolas *et al.* 2014). While APP knockout mice show no significant phenotype, APLP2/APLP1 or APLP2/APP double knockout mice were non-viable, likely owing to the redundancy between the 3 similar proteins (Heber *et al.* 2000). In human embryonic stem cells, like in mice embryos, it has been shown that APP is able to induce neural differentiation towards a neuronal phenotype (Freude *et al.* 2011). Interestingly, APP interaction at synapses has been shown to regulate synaptic activity (Wang *et al.* 2009). Unfortunately, despite all these possibilities there has still been no

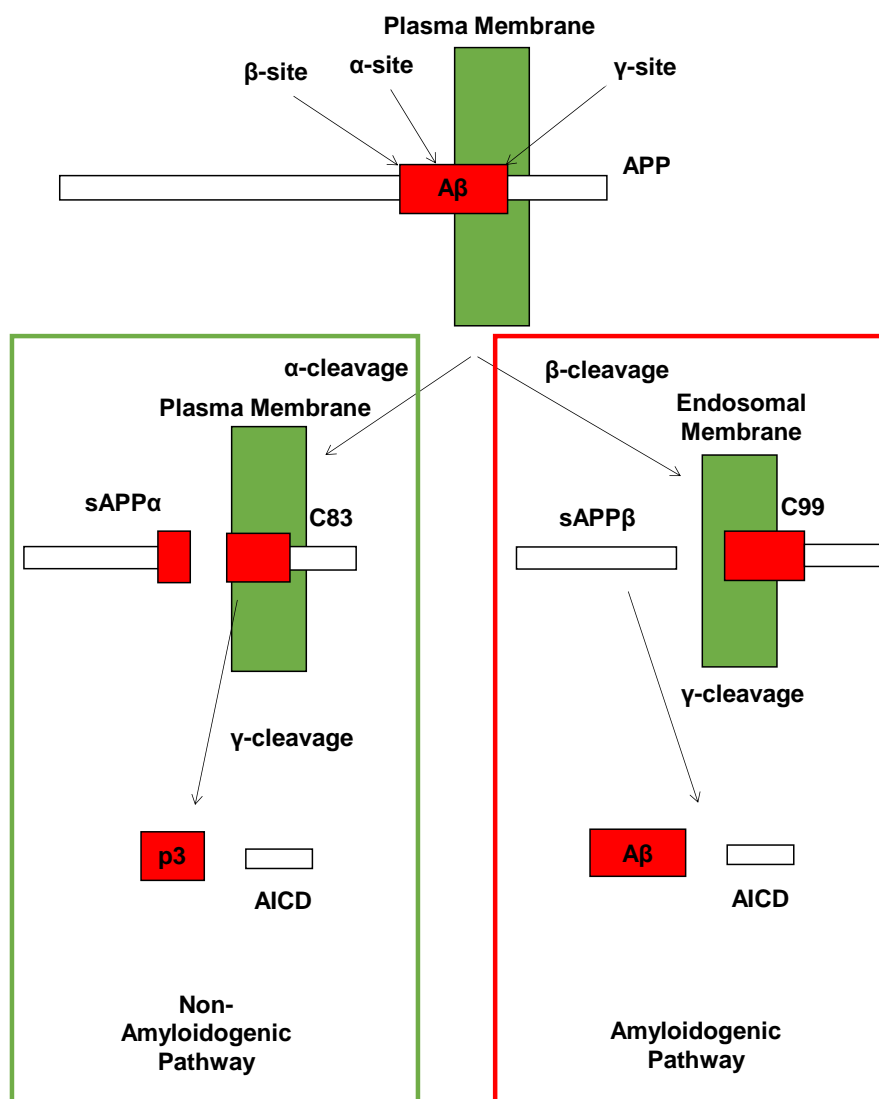


Figure 1. *Pathways for APP processing.* The Aβ domain is highlighted in red. The amyloidogenic pathway is encompassed by a red box, while the non-amyloidogenic pathway is enclosed by a green box. In the amyloidogenic pathway, the β-secretase first cleaves APP on the endosomal membrane at the β-cleavage site producing sAPPβ and C99. The C-terminal fragment C99 is then further cleaved by γ-secretase in an unknown compartment to yield the 36-43 amino acid long Aβ peptide and the AICD. In the non-amyloidogenic pathway, α-secretase instead cleaves APP on the plasma membrane at the α-cleavage site, in the middle of the Aβ domain, producing sAPPα and C83. Further γ-secretase cleavage at an unknown compartment yields the non-toxic p3 and the AICD.

definitive answer for the function of APP. The downstream products of APP may also have their own physiological functions, such as AICD and soluble APP fragments, sAPP α and sAPP β , however their possible roles will be discussed further below.

1.2.2.2 APP in Alzheimer's Disease

A number of mutations in the APP gene have been shown to be responsible for many cases of Familial early onset Alzheimer's Disease (FAD). These mutations are divided into three classes and all these classes of mutations affect A β generation or deposition. The first class of mutations is located near the β -cleavage site of APP. One notable example from this class, the Swedish mutation, is located on the APP gene adjacent to the location of the β -cleavage site, causing an increase in A β production by 10-fold (Mullan *et al.* 1992). The second class, of mutations are located near the location of the γ -cleavage site of APP, and increase the relative amount of A β 42 thereby increasing the A β 42/A β 40 ratio. The London mutation is an example of a mutation belonging to this class (Goate *et al.* 1991; Hendriks *et al.* 1992; Eckman *et al.* 1997). There are also mutations near the alpha site that may decrease α -cleavage or increase aggregation of A β . Some examples of mutations from this class are the Arctic, Dutch, and Iowa mutations (Van Nostrand *et al.* 2001; Nilsberth *et al.* 2001; Cheng *et al.* 2004). Complete duplications of chromosome 21 in humans that houses the APP gene, which is observed in people with Down's syndrome, also results in AD pathogenesis much earlier in life (Cabrejo *et al.* 2006). However, duplications of only the APP gene have been seen in rare families with FAD (Rovelet-Lecrux *et al.* 2006; Sleegers *et al.* 2006). In summary, the main effect of APP in Alzheimer's disease is the generation of A β , and whether APP has other roles in Alzheimer's disease pathogenesis outside of this is not known.

1.2.3 Normal Physiological Function of A β

The A β peptide was sequenced and identified as an Alzheimer's disease marker more than three decades ago (Glennner *et al.* 1984). Since then, A β has been studied extensively with regards to its production in the hopes that halting this process could provide a possible cure for Alzheimer's disease. However, the physiological role of A β and its purpose are still unclear.

The non-amyloidogenic route of APP processing produces sAPP α and C83 (See Figure 1). While not entirely understood, there have been numerous studies looking at the role of sAPP α showing its ability to promote long-term potentiation (LTP), improve spatial memory, as well as playing a role in neuroprotection (Taylor *et al.* 2008; Chasseigneaux *et al.* 2012). It is also possible that sAPP α may play an antagonistic role to A β , providing a possible neuroprotective effect (Hartl *et al.* 2013). Similarly, the role of C83 is not known, however C83 is further cleaved to release p3 and an amyloid intracellular domain (AICD). The role of p3 itself is not known, however AICD plays an important transcriptional role. It has been found that AICDs associate with FE65, and the AICD-FE65 bound complex must be liberated from APP through sequential cleavage before it can translocate to the nucleus and bind to transcription factors there (McLoughlin *et al.* 2008).

In the amyloidogenic pathway, the first products are sAPP β and C99. The two proteins sAPP β and sAPP α seemingly share identical functions; however, sAPP β is 16 amino acids shorter on the C-terminal. With regards to neuroprotective function and long-term potentiation, sAPP β has greatly reduced potency when compared to sAPP α (Barger *et al.* 1997; Taylor *et al.* 2008; Chasseigneaux *et al.* 2012). Therefore, it has been hypothesized that the 16 amino acid C-terminal that is truncated during β -cleavage is highly involved in both neuroprotection and LTP. As for C99, while a physiological role isn't clear, a transgenic mouse expressing human C99 was shown to have increased acetylcholinesterase (AChE) activity in the medial septum of the brain, correlating with poor spatial learning, as tested by a Morris water maze (Dumont *et al.* 2006). Interestingly, it has been demonstrated that α -secretase has the ability to cleave C99,

converting it to C83 and preventing further A β generation (Jäger *et al.* 2009). C99 is further cleaved to produce A β and once again AICD, whose function has already been discussed.

The normal function of A β is unknown. A β has been shown as having a possible neuroprotective effect in neurons at very low doses (Kamenetz *et al.* 2003; Plant *et al.* 2003). Furthermore, this neuroprotective effect is based on A β peptide size, with some studies showing that A β 40 provides a neuroprotective effect in a concentration-dependent manner, and other A β forms showing almost no effect. Furthermore, it has been demonstrated that the A β peptide may be necessary for learning at physiologically low doses (Morley *et al.* 2012). Puzzo *et al.* (2011) found that depletion of endogenous A β impaired hippocampal LTP and learning, but injections of A β 42 at picomolar concentrations, similar to those normally present in the body, were able to rescue the impairment in memory. Additionally, similar results were reported in another study, with picomolar concentrations of A β improving memory (Morley *et al.* 2014). A β has also been suggested to be able to regulate K⁺ and Ca²⁺ channel activity (Ramsden *et al.* 2002; Plant *et al.* 2006), act as an antimicrobial peptide (Soscia *et al.* 2010), and regulate cholesterol transport (Igbavboa *et al.* 2009; Yao *et al.* 2002). Contrary to the above however, it has also been shown that higher levels of A β actually impairs memory and cognitive function (Cleary *et al.* 2005; Ozdemir *et al.* 2013; Chambon *et al.* 2011). A β has also been shown to stimulate synaptic depression, providing an additional role in cognitive decline (Kamenetz *et al.* 2003). Furthermore, A β has been shown through numerous studies to be toxic to synapses and neurons in AD (Walsh *et al.* 2007). These studies provide a role for A β in the pathogenesis of Alzheimer's Disease, which will be discussed more fully below. However, despite all these possibilities there is no definitive answer as to what role A β may play in normal physiology.

1.2.4 Amyloid Beta and Alzheimer's Disease

It has long been known that the A β peptide is the principal component of amyloid plaques from previous studies looking at amyloid deposits in the brains of AD and Down's syndrome patients (Masters *et al.* 1985; Glenner *et al.* 1984). As mentioned above, this link between AD and Down's syndrome was solidified through studies that showed pathological features in Down's syndrome patients that were extremely similar to those in AD, but at a much earlier age (Wisniewski *et al.* 1985). Specifically, Down's syndrome brains showed atrophy of the brain, as well as early growth arrest that is not present in AD brains. Furthermore, localization of the APP gene points to chromosome 21, the chromosome that is duplicated in Down's syndrome (Trisomy 21) (Tanzi *et al.* 1987). These results lead to the idea that increased APP expression, which would occur in the case of an additional copy of chromosome 21, leads to increased A β deposits in the brain, and prompted study into its neurotoxic effects. Another link between APP, A β and Alzheimer's disease came from studies done in FAD, which was already discussed above. Further evidence came from β -secretase and γ -secretase themselves. β -secretase has been shown to have both increased activity and protein expression in patients with Alzheimer's disease (Fukumoto *et al.* 2002; Holsinger *et al.* 2002). On the other hand, γ -secretase mutations have been shown to be present in some FAD families, with presenilin-1 (PS1) mutations comprising the largest group of FAD, but how these presenilin-1 mutations affect γ -secretase activity is unknown. However, they appear to cause Alzheimer's disease by increasing the A β 42/A β 40 ratio (Tam *et al.* 2012). This can occur through increased A β 42 production in relation to A β 40, or even decreasing A β 40 production with no changes in A β 42 production; some PS1 mutations even lower total A β production, with a greater decrease in A β 40 (Sheuner *et al.* 1996; Shimojo *et al.* 2007; Wolfe 2007).

Due to the strong association between A β and Alzheimer's disease, much work has been done to understand the pathological role that amyloid itself plays in neurodegeneration and cognitive decline in AD. Of the two major A β forms, A β 42 aggregates much more easily than A β 40. This was observed by Bitan *et al.* (2003) where they observed differences aggregation profiles. While A β 40 was able to quickly form dimers, trimers,

and tetramers, only A β 42 aggregated to form large oligomers. Furthermore, the ratio of A β 42 to A β 40 has been linked to the mean age of onset in Alzheimer's disease patients strengthening support for A β 42 as the most toxic isoform (Duering *et al.* 2005). A β oligomers themselves have been shown to disrupt synaptic plasticity, and inhibit long-term potentiation at physiologically relevant concentrations, contrary to what was stated previously (Shankar *et al.* 2008; Walsh *et al.* 2002). The reason for the discrepancy between these two lines of evidence is not clear, however a possible reason is stated in the study by Walsh *et al.* (2002), stating that A β oligomers are the cause for LTP inhibition, not A β monomers. With the use of insulin-degrading enzyme (IDE), that only degrades A β monomers and not oligomers, they were not able to prevent the inhibition of LTP. Most importantly, A β oligomers are able to trigger synapse loss and neuronal death (Deshpande 2006; Lambert *et al.* 1998; Shankar *et al.* 2007). However, another possible pathological role for A β 42 is in oxidative stress in the brain, contributing to neuronal death (Weidner *et al.* 2011; Butterfield *et al.* 2002). A β is able to induce lipid peroxidation impairing ion channels, glucose and glutamate transports, and guanosine triphosphate (GTP) - binding proteins (Mattson 2004). This oxidative activity may occur through the regulating aldehyde 4-hydroxynonenal (HNE) which conjugates to and alters the activity of proteins (Mark *et al.* 1997). A β treatments in SK-N-BE cells have also been shown to activate c-Jun amino terminal kinase (JNK) and p38 (MAPK), a pair of stress-activated protein kinases that have been shown to demonstrate activity in neuronal cells undergoing apoptosis (Tamagno *et al.* 2003).

1.2.5 Amyloid Cascade Hypothesis

Taken together, the foregoing findings provide a model for how A β may lead to the synapse loss, neuronal death, and cognitive impairment characteristic of Alzheimer's disease, a pathway referred to as the amyloid cascade hypothesis. In this hypothesis it is postulated that increasing relative A β 42 generation, above natural levels of clearance in the brain, will cause accumulation of A β and initiate Alzheimer's disease pathogenesis. In the original form of the Amyloid hypothesis, A β 42's ability to easily aggregate would

result in rapid deposition occurs and plaque formation. Plaques were believed to alter neuronal homeostasis and alter kinase and phosphatase activity, while promoting generation of neurofibrillary tangles leading to the progression of dementia and eventually, neuronal death (Hardy *et al.* 2002). However, there were a few problems that the old amyloid cascade hypothesis was faced with. Of primary concern was that amyloid plaque presence does not correlate well with cognitive function in humans, and similarly, many mouse models with progressive A β deposition do not exhibit definitive neuronal loss (Tam *et al.* 2012; Hardy *et al.* 2002). This point of contention may be explained through the recent study of Amyloid Derived Diffusible Ligands (ADDLs), or large soluble oligomers of A β . ADDLs have been shown to correlate well with synaptic loss (Tomic *et al.* 2009), and may provide an explanation for why patients with Alzheimer's disease may show relatively little amyloid deposition. Furthermore, as discussed previously, A β oligomers are orders of magnitude more toxic than the fibrils found in plaques, inducing synapse loss and neuronal death at much lower concentrations (Walsh *et al.* 2007).

The generation of neurofibrillary tangles (NFTs), composed of aggregated tau protein, downstream of A β 42 activity may serve to exacerbate this process further. NFTs are present in other forms of dementia such as frontotemporal dementia. After suppression of tau it has been shown that memory function recovers and fails to worsen further. (Santacruz *et al.* 2005). Furthermore, it has been shown that A β 42 is able to promote the phosphorylation and aggregation of Tau, promoting Tau pathology, while A β 40 actually decreased Tau phosphorylation (Hu *et al.* 2014). This may reinforce the hypothesized validity of the A β 42/A β 40 ratio by providing a mechanism for why having more A β 40 relative to A β 42 may be beneficial.

1.2.6 APP Trafficking

One important aspect of APP that regulates A β production is its trafficking. APP is synthesized in the Rough Endoplasmic Reticulum (RER), exits its site of production, and

is immediately transported to a number of locations intracellularly. The first site is the Golgi, where APP undergoes post-translational modifications such as *N*- and *O*-glycosylation, phosphorylation, and tyrosine sulfation. While much APP at steady state localizes to the Golgi and the trans-Golgi network (TGN), a major site of transport for APP is the plasma membrane (Thinkaran *et al.* 2008). This is where α -cleavage of APP may first occur, as well as the location where the adaptor protein FE65 can bind to the C-terminal end of APP (Sisodia 1992; McLoughlin *et al.* 2008). The binding of FE65 to a YENPTY motif at the C-terminal of APP causes APP to be rapidly transported from the plasma membrane back into the cell (Thinkaran *et al.* 2008). From here, APP moves back into the cell through endocytosis towards the endosomal/lysosomal system which has been shown to be necessary for A β production, as BACE1 and the γ -secretase complex are localized to endosomes (Koo *et al.* 1994; Thinkaran *et al.* 2008). As further evidence to the importance of APP internalization to A β production, it has also been found that YENPTY mutations can inhibit APP internalization and decrease A β generation (Perez *et al.* 1999). While much APP does localize to the Golgi/TGN, we have recently discovered that APP is also able to traffic from the Golgi to lysosomes through adaptor protein 3 (AP-3), a lysosomal trafficking protein (Tam *et al.* 2014).

1.3 Endosomal/Lysosomal System

There have been many studies done suggesting a pivotal role for the endosomal/lysosomal system in the production of A β , where APP cleavage has been shown to occur after endocytosis from the cell surface (Pasternak *et al.* 2004). Before further discussing the role of the endosomal/lysosomal system in A β production, an overview of system itself will first be provided (See Figure 2).

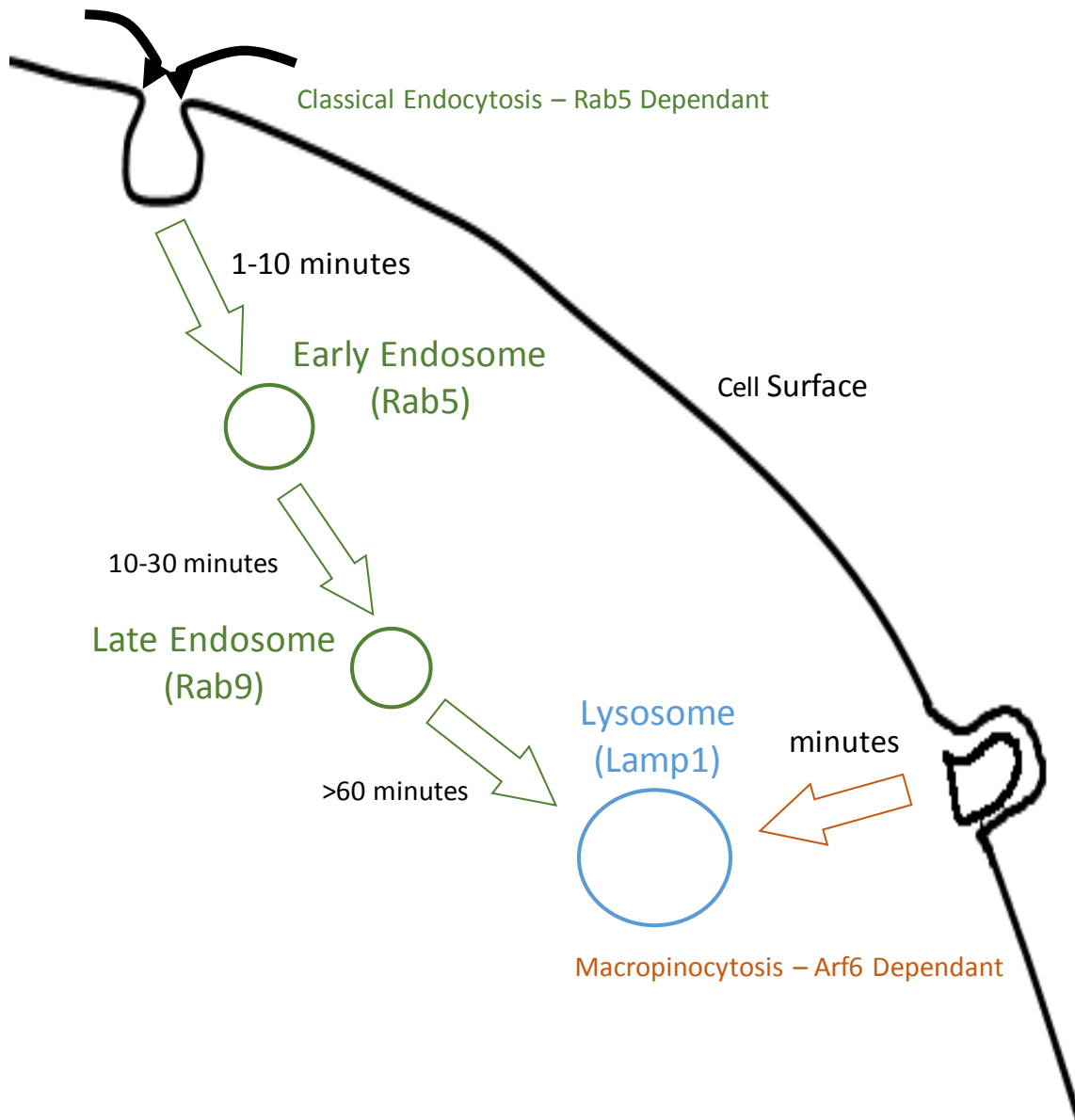


Figure 2. *Routes for the internalization of APP to lysosomes.* The best described pathway of APP internalization occurs through classical clathrin-mediated endocytosis that occurs through a Rab5-dependant mechanism. APP is first endocytosed into an early endosome, which matures to a late endosome, before finally fusing with a lysosome. The entire process from endocytosis to fusion with the lysosome can occur over hours. We have discovered a second pathway for APP internalization we have found occurs through what is believed to be macropinocytosis directly from the cell surface producing a macropinosome that goes on to fuse with a lysosome. This process occurs very quickly within minutes.

1.3.1 Endosomes

Endosomes are small intracellular membrane-bound compartments that play a pivotal role in the sorting and recycling of membrane components. This has effects on a number of basic cellular processes, such as nutrient uptake, immunity, signaling, and development (Scott *et al.* 2014). The materials within endosomes must first enter the cell through a process named endocytosis. Endocytosis is defined as the internalization of the plasma membrane, associated ligands, and the surrounding fluid (Hansen *et al.* 2009).

These endocytosed materials are commonly moved to an early endosome after endocytosis from the cell membrane. Early endosomes serve as a sorting station, allowing components that need to be moved to be recycled back to the plasma membrane, transported towards the TGN, or transported towards lysosomes can all be separated (Scott *et al.* 2014). Early endosomes (EE) eventually mature into late endosomes which may transport endocytosed materials to the TGN or fuse with lysosomes. These two major classes of endosomes, while serving a similar purpose, can be distinguished between using the proteins they associate with. Early endosomes are closely associated with the regulatory protein Rab5 along with its effector VPS34/p150, that generates phosphatidylinositol 3-phosphate (PI(3)P). Rab5 also plays an important role later on as the main regulator of conversion from early endosomes to late endosomes. Maturation of early endosomes occurs through its recruitment of the protein Rab7 to the endosomal membrane, which serves to exchange the fusion machinery of the maturing endosome to ensure fusion of the late endosome only occurs with other late endosomes, lysosomes, and the TGN (Huotari *et al.* 2011). Late endosomes are later strongly associated with Rab9, a small GTPase that is essential for the retrograde pathway of transport from late endosomes to the TGN, but not the anterograde pathway, from late endosomes to the lysosome.

In summary, early endosomes and late endosomes can be differentiated between by which Rab is presented at their membranes. Early endosomes present Rab5, while late endosomes present Rab9 at their membranes. While Rab7 is also present at late endosomes, as discussed above it is recruited to early endosomes as they mature into late

endosomes as well, so for the purposes of differentiation, Rab9 is a better marker (Lu *et al.* 2014). Late and early endosomes may also be differentiated by their luminal pH. Early endosomes typically have a pH ranging from 6.8 to 6.1, while late endosomes show a pH in the range of 6.0 to 4.8. This distinguishes them from lysosomes, which can have pH values as low as 4.5 (Maxfield *et al.* 1987). This lowered luminal pH serves multiple purposes in the endosomal system such as inactivation of pathogens, in membrane trafficking, and the further sorting of cargo. The lowered pH also serves as a better environment to better facilitate ligand-receptor dissociation on the membrane, allowing receptors to be recycled back to the plasma membrane (Huotari *et al.* 2011). Another marker of late endosomes is Mannose-6-phosphate receptors (M6PR). M6PR traffics between the late endosome and the TGN through the packing of lysosomal hydrolases tagged with M6P facilitated by binding to the M6PR. The hydrolases are then transported to the late endosomes where the luminal pH causes dissociation between M6P and the M6PR. The M6PR is then recycled to the TGN to once again bind M6P (Lu *et al.* 2014).

1.3.2 Lysosomes

In the 1950s lysosomes were described as the major digestive compartment of cells by Christian de Duve and his group (Appelmans *et al.* 1955; de Duve 2005). From studies performed in rat livers he suggested the existence of a membrane-bound compartment with a low pH containing a number of hydrolytic enzymes, dubbing it the lysosome (de Duve 2005). Since then, lysosomes have been considered to be the garbage cans of the cell, simply a place for damaged proteins, unneeded signaling molecules, and other cellular parts to go for breakdown and eventually recycling of their materials. However, as more research into lysosomes occurs it has been revealed that lysosomes are necessary for many cellular processes important to homeostasis aside from degradation such as plasma membrane repair, cholesterol homeostasis, and the regulation of apoptosis. The importance of the lysosome to homeostasis is also highlighted by the fact that there are a group of over 40 diseases related to the lysosome, referred to as lysosomal storage diseases. These diseases occur through the accumulation of metabolic end products

within the lysosome as a result of either missing or impaired function of lysosomal enzymes (Pasternak *et al.* 2004). In terms of diseases related to lysosomal trafficking, there exists at least one example, a rare condition called Chédiak-Higashi syndrome. In this extremely rare disease, a mutation in the LYST protein, a regulator of lysosomal trafficking, results in enlarged lysosomes and heavily impaired immune function (Kaplan *et al.* 2008).

Material for degradation in the lysosomes arrives through multiple pathways. The first pathway, endocytosis, sees the fusion of a late endosome with a lysosome to form a secondary lysosome, which then fuses with more lysosomes later. Late endosomes targeted for the lysosomal degradation pathway and lysosomes themselves are enriched in marker Rab7 (but not Rab9 like in late endosomal transport to the TGN) and the lysosomal-associated membrane protein 1 (LAMP-1), a transmembrane protein showing high expression in lysosomes. Lysosomes also lack the mannose-6-phosphate receptor (M6PR), which is present in late endosomes (Appelqvist *et al.* 2013). As discussed above, the pH of late endosomes is quite low, and decreases as it fuses with lysosomes, eventually reaching standard lysosome pH of 4.5. This decrease in pH is facilitated by ATP dependent proton pumps (Vacuolar ATPases) on the membrane that lower luminal pH of the vesicles. This low pH is necessary for the hydrolytic function of the proteases within the lysosome, which function optimally in highly acidic environments (Appelmans *et al.* 1955; Coffey *et al.* 1968). The second pathway, autophagy, transports cytoplasmic proteins, whole organelles, cytoplasm, and anything within the cytoplasm directly to lysosomes for degradation. In immune cells, phagocytosis and macropinocytosis also transport extracellular material to the lysosome for degradation. The entire process from early endosome formation to lysosomal fusion takes approximately 40 minutes (Appelqvist *et al.* 2013). The process of degradation in the lysosome is achieved by ~60 different hydrolases. These hydrolases include proteases, peptidases, phosphatases, nucleases, glycosidases, sulfatases, and lipases. Of these hydrolases some of the best known ones are from the cathepsin family, being divided into three different groups based on the active site amino acid. These are serine (including cathepsin A and G), aspartic acid (cathepsin D and E), and cysteine (cathepsins B, C, F, H, K, L, O, S, V, W

and X). Of the above, the most abundant and ubiquitous of the cathepsins in lysosomes are: cathepsin B, C, D, H, and L (Rossi *et al.* 2004).

More recently, lysosomes were demonstrated to have secretory function as well, dispelling the myth that lysosomes only served as the endpoint for endocytosed materials. In a study conducted by Rodriguez *et al.* (1997) it was found that lysosomes were able to secrete calcium in both fibroblasts and epithelial cells, not just specialized secretory cells, in response to high intracellular calcium. This lysosomal secretion appears to be regulated by the GTPase, Rab27, which has two isoforms, shown to regulate exocytosis of lysosome-related organelles in a number of cells (Izumi *et al.* 2003; Izumi 2007). From there the lysosome fuses with the membrane, releasing its contents extracellularly (Jaiswal *et al.* 2002). This recent change in the understanding of lysosomes has revealed several pivotal roles for lysosome secretion. In osteoclasts there is evidence suggesting that lysosomes through exocytosis are able to transport vacuolar type H⁺-ATPase (V-ATPase) to the plasma membrane. Once there, V-ATPase causes extracellular acidification, mobilizing bone calcium (Toyomura *et al.* 2003). Lysosomes also play a role in cell membrane repair, as Reddy *et al.* (2001) showed that elevation of intracellular calcium, which would occur in plasma membrane damage, is required for lysosome exocytosis, further demonstrated in the resealing of primary skin fibroblasts through lysosomal exocytosis. This corroborates the work done earlier by Rodriguez *et al.* discussed previously, showing that that high intracellular calcium stimulated lysosome secretion. Additionally, lysosome exocytosis has been shown to also regulate ATP release in astrocytes, with ablation of ATP secretion occurring after lysis of lysosomes (Zhang *et al.* 2007). Lysosomal secretion may also be vital in the presentation of MHC Class II-antigen complexes at the plasma membrane (Andrews 2000).

1.3.2.1 Lysosomes and Alzheimer's Disease

As the roles of lysosomes in the cell and as a regulator of normal physiology expand an increasingly large number of diseases have been linked to lysosomal dysfunction. A

number of studies in Alzheimer's disease have noted a connection between AD and altered lysosomal function, as well as A β production (Nixon *et al.* 2011; Lee *et al.* 2010; Coen *et al.* 2012). Starting with the internalization of APP, studies have shown for a long time that that A β generation requires the normal functioning of endocytosis (Cirrito *et al.* 2008; Koo *et al.* 1994). Furthermore, de-acidification of this system causes significant reductions in the production of A β , as shown through the application of the alkalizing agents ammonium chloride (NH₄Cl), bafilomycin A1 (bafA1), chloroquine, and concanamycin A (Schrader-Fischer *et al.* 1996; Vingtdeux *et al.* 2007). The previous study mentioned by Schrader-Fischer and Paganetti (1996) showed the accumulation of APP intracellularly after the application of alkalizing agents. However, C-terminal generation of the C99 was completely inhibited. As C99 is generated through the initial β -cleavage of APP it is possible that de-acidification of endosomes may halt APP generation at the β -cleavage step. However, APP processing inhibition from de-acidification may also occur at the γ -cleavage step.

We have found previously that nicastrin, a major lysosomal membrane protein, APP and presenilin-1 all colocalize strongly with LAMP1. Additionally, in the same study γ -secretase demonstrated optimal activity in highly acidic environments, similar to what would be present in the lysosome (Pasternak *et al.* 2003). As further evidence for lysosomes as the location of γ -secretase activity, in PS1(-/-) murine neurons, the COOH-terminal fragment (CTF) which is eventually cleaved by γ -secretase also showed accumulation in the lysosomes as a result of loss of function in PS1 (Chen *et al.* 2000). Lastly, a number of studies have shown that A β is secreted in exosomes, which are intraluminal vesicles of the late endosome/lysosome (Rajendran *et al.* 2006; Vingtdeux *et al.* 2007).

Apart from A β generation, it has also been suggested that lysosomes may also play a role in the aggregation of A β to form the fibrils present in amyloid plaques. It has been known for over a decade that A β fibrillogenesis occurs optimally at acidic pH levels, a well-known characteristic of the lysosome; even at mild pH levels, like those seen in endosomes, A β aggregates begin to form, albeit to a lesser degree than at a lower pH (Su *et al.* 2001; Inouye *et al.* 2000; Gorman *et al.* 2003). In addition, gangliosides present in

the plasma membrane of lysosomes in tandem with a low pH have also been shown to accelerate A β aggregation, as well as promote membrane disruption (McLaurin *et al.* 1996; Waschuk *et al.* 2001). Aggregation of A β has even been shown to occur upon the application of exogenous A β 42, with markers pointing to the late endosome or lysosome as one of the major sites of accumulation and aggregation (Burdick *et al.* 1997; Knauer *et al.* 1992). A β fibrils themselves have demonstrated the ability to disrupt lipid membranes at synapses and in lysosomes, initiating leakage of lysosomal contents resulting in cell death (McLaurin *et al.* 1996; Yang *et al.* 1998; Ji *et al.* 2002). The connection between lysosomal leakage and cell death may come from either apoptosis initiated by activation of caspase proteases, from the release of lysosomal hydrolases into the cell, or from the liberated A β itself (Zhang *et al.* 2007).

Despite all the evidence provided, lysosomes have yet to be confirmed as the definitive site for A β production in Alzheimer's disease. The Golgi, plasma membrane, and ER have also been suggested as possible sites by other authors. However, the lysosome still provides possibly the best location for a major site of A β production, and so we believe that by studying the pathways that transport APP to the lysosome we may be able to eventually find therapies that work for Alzheimer's disease. For example, since APP is required to undergo endocytosis for the generation of A β it may be possible to inhibit APP trafficking at this first step, thereby eliminating the progression of the amyloid cascade and providing a future avenue for therapy in Alzheimer's disease patients.

1.4 Endocytosis

Endocytosis can be divided into two basic categories, clathrin-dependent endocytosis, or clathrin-mediated endocytosis (CME), and clathrin-independent endocytosis (CIE).

1.4.1 Clathrin-mediated endocytosis

Clathrin-dependent endocytosis, also referred to as classical endocytosis, is the best understood process. Clathrin-dependent endocytosis generates small (~50 nm - ~100 nm diameter) vesicles coated with the protein clathrin from which this process derives its name. The first step of clathrin-mediated endocytosis is assembly of clathrin into a polygonal lattice and the formation of coated pits on the plasma membrane that is promoted by adapter protein-2 (AP-2) (Merrifield *et al.* 2014). In these coated pits, clathrin assembles into a shape referred to as a triskelion, reminiscent of a spiral with 3 arms. This assemblage consists of three heavy and three light clathrin chains bound together. The clathrin heavy chain has been suggested to be important for binding to AP-2, while the light chain has been suggested to regulate the actual formation of the clathrin assembly (Mousavi *et al.* 2004). AP-2, along with other adapter proteins, binds the cytosolic tails of membrane proteins, and is essential for localization of the clathrin onto the plasma membrane. More specifically, AP-2 binds phosphatidylinositol-4, 5-bisphosphate (PIP₂), an important signaling molecule for multiple modes of endocytosis, causing a conformational change to reveal cargo and clathrin-binding motifs. When the AP-2 complex then successfully binds receptor cargo and clathrin, nucleation of a clathrin-coated pit is initiated (Jackson *et al.* 2010). The clathrin-coated pits invaginate further until only a “neck” remains connecting a fully enclosed clathrin-coated vesicle. This neck is then severed by a large GTPase named dynamin thereby releasing the clathrin-coated vesicle from the plasma membrane (Merrifield *et al.* 2014). At this point vesicles are able to fuse with early endosomes and thought to be sorted for recycling or trafficking to late endosomes as previously discussed. The fate of the vesicle after this is movement to the lysosome or TGN. Generally, the entire process for transport to lysosomes or the TGN may take up to an hour, with proteins showing in early endosomes as early as 10 minutes, and in late endosomes as early as 30 minutes.

1.4.2 Clathrin-independent endocytosis

The clathrin-independent class of endocytosis encompasses multiple different pathways. These pathways are macropinocytosis and endocytosis, for large vesicles ($>1\ \mu\text{m}$), and the caveolar, flotillin, and CLIC/GEEC pathways for smaller vesicle sizes. The microscale pathways for clathrin-independent endocytosis will be discussed here, while phagocytosis and macropinocytosis will be discussed further on.

1.4.2.1 Caveolar Pathway

Of the dynamin-dependent pathways of CIE, the caveolar one is the best studied. Caveolae are 50-80 nm wide, flask-shaped plasma membrane invaginations rich in caveolin, as its name suggests. In addition to caveolin, caveolae are enriched in sphingolipids, cholesterol, signaling proteins, and clustered glycosyl phosphatidylinositol-anchored proteins (GPI-Aps). The caveolins were first discovered with the identification of caveolin-1 (CAV1) over two decades ago, after which caveolin-2 (CAV2) and caveolin-3 (CAV3) were also identified (Rothberg *et al.* 1992). CAV1 and CAV2 are only present in non-muscle cells, whereas CAV3 localizes to skeletal muscle and to a limited degree in smooth muscle cells (Parton *et al.* 2007). CAV1 and CAV3 ablation causes the loss of caveolae, while no similar effect is seen with CAV2 ablation. Although it is possible that because CAV1 and CAV2 are present in the same types that there is redundancy between the two proteins (Drab *et al.* 2001; Galbiati *et al.* 2001; Razani *et al.* 2002). Caveolae form through the oligomerization and association of caveolin with cholesterol-rich lipid-raft domains. As a result of the tight association between the caveolin protein and cholesterol, cholesterol depletion has been shown to cause the disassembly of caveolae (Rothberg *et al.* 1992). Endocytosis of caveolae waiting at the plasma membrane is initiated by a number of different ligands ranging from albumin, to viruses, and even sterols and glycosphingolipids (Pelkmans *et al.* 2002; Sharma *et al.* 2004; Minshall *et al.* 2002). Budding of the caveolae itself is regulated by the Src-family kinases, local actin polymerization, and dynamin, hence its classification

under dynamin-dependent CIE (Sverdlov *et al.* 2007). Unfortunately, study of caveolar mechanisms as well as those in dynamin-dependent, caveolin-independent pathways has been made difficult by a number of problems. First, endocytic cargo that travels through caveolin-dependent pathways can also be internalized through different mechanisms in other cell types, or even the same cell itself. Secondly, other lipid-raft endocytosis pathways that are clathrin- and caveolin- independent have been discovered, confounding the study of other clathrin-independent forms of endocytosis (Mayor *et al.* 2007).

1.4.2.2 Flotillin Pathway

The flotillin-dependent was first suggested after the observation of residual caveolar structures despite caveolin-1 depletion in mice models for knockout of the caveolin-1 gene (Zhao *et al.* 2002). It was not until later that flotillin 1 and 2, proteins that are associated with the membrane in caveolae, were observed to generate caveolar-like structures independent of both caveolin and clathrin (Glebov *et al.* 2006; Frick *et al.* 2007). However, in mouse embryonic fibroblasts with a knockout for caveolin-1, but also overexpressing flotillin 1 and 2, caveolae were not generated (Kirkham *et al.* 2008). Therefore, the flotillin-mediated pathway is not currently a definitive pathway for CIE.

1.4.2.3 CLIC/GEEC Pathway

The best-described clathrin-independent, dynamin-independent pathway is the clathrin-independent carriers/ GPI-AP enriched early endosomal compartments (CLIC/GEEC) pathway. The carrier for this pathway is a 100-200 nm large ring-shaped compartment (Kirkham *et al.* 2005). This pathway was first identified in a study of HeLa cells in which impairment of dynamin-1 increased fluid-phase uptake through a clathrin-independent pathway (Damke *et al.* 1995). The implication of Cdc42 in this pathway was later discovered in a study following the internalization of glycosylphosphatidylinositol-

anchored proteins (GPI-APs). In this study no change was observed in GPI-AP endocytosis after RhoA, dynamin 2, or Rac1 inhibition. However, Cdc42 inhibition was able to diminish fluid-phase uptake and redistribute GPI-APs to clathrin-mediated endocytosis (Sabharanjak *et al.* 2002). This pathway has been shown to also be the main endocytic route for cholera toxin B, *Helicobacter pylori* vacuolating toxin, and the GPI-anchored cellular prion protein (PrP^C) (Mayor *et al.* 2007). Additionally, Arf1 has been shown to also be involved in regulation of this pathway, and because of the substantial involvement of Arf1 and Cdc42 it has been suggested that this pathway may also be regulated through other key proteins in actin dynamics (Kumari *et al.* 2008).

1.4.3 Macroscale Endocytosis

1.4.3.1 Phagocytosis

Phagocytosis was initially discovered by Metchnikoff over 100 years ago (Flannagan *et al.* 2012). Since then, numerous roles for phagocytosis have been uncovered aside from the adaptive immune response to pathogens; this includes tissue homeostasis and remodeling. Phagocytosis is by definition the ingestion of particles greater than 0.5 μm in diameter. Phagocytosis differs from the other form of macroscale endocytosis (macropinocytosis) because it is directed through cell surface binding directly to target particles or foreign bodies causing projections to protrude from the membrane, encircling and engulfing the foreign object. After total enclosure of the object and scission from the membrane the compartment is called a phagosome. The phagosome then moves intracellularly, fusing with a lysosome to degrade the luminal contents of the phagosome. Through this they have well-documented roles in immune defense, and even the ingestion of apoptotic bodies in non-immune cells (Flannagan *et al.* 2012). As phagocytosis functions in such a broad role, there are many receptors that have been found and studied in the initiation of phagocytosis.

Phagocytosis is triggered by receptor binding at the cell surface. Most of these receptors can be separated into three main categories: Fc receptors, integrins, and a very diverse third group, the pattern-recognition receptors which are not well understood but include Dectin-1 receptors and scavenger receptors (Freeman *et al.* 2014).

The first group, the Fc receptors, facilitate phagocytosis through the binding of opsonized-immunoglobulin G (IgG) particles (Indik *et al.* 1991), and provides a mechanism for clearing antibody-bound pathogens/targets. There are six different human Fc receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB) that have the ability to bind to multiple different IgGs with differing affinities for each (Bruhns *et al.* 2009). Once bound to immunoglobulin, Fc receptors initiate phagocytosis through actin polymerization at the plasma membrane. Actin polymerization is facilitated by recruitment of the Arp2/3 complex to the phagocytic cup. The Arp2/3 complex is normally inactivated and must be activated by neural Wiskott-Aldrich syndrome protein (N-WASp) in order for actin nucleation to begin (Park *et al.* 2009; Lorenzi *et al.* 2000; Tsuboi *et al.* 2007). However, N-WASp must also be activated itself before it can activate bind and activate the Arp2/3 complex. This is achieved through the binding of the small GTPase, Cdc42, as well as phosphatidylinositol 4,5-bisphosphate (PIP2), a small phospholipid which is an important molecule in many types of endocytosis as mentioned previously (Prehoda *et al.* 2000). Other actin dynamic regulators that have been shown to play a role are Rac1 and Rac2, Arf6, and even RhoA (Hoppe *et al.* 2004; Hall *et al.* 2006; Zhang *et al.* 1998; Jankowski *et al.* 2008; Beemiller *et al.* 2006). Interestingly, the WASP family Verprolin-homologous (WAVE) complex, which performs a similar role to N-WASp in the activation of the Arp2/3 complex and initiation of actin nucleation, has been shown to not be necessary for Fc receptor-mediated phagocytosis (Kheir *et al.* 2005). To add to this, the WAVE complex is activated downstream of both Rac1 and Arf6 as well. However, despite linking the proteins above to FcγR-mediated phagocytosis, their exact role and their effectors in this process are unknown. After engulfing the IgG-bound particle actin polymerization is halted through currently unknown means, although some studies suggest phosphatidylinositol 3-kinase (PI3K) may regulate this process (Freeman *et al.* 2014). The compartment that is released from

the membrane is called a phagosome, eventually fusing with a lysosome to degrade the contents within.

The second group of receptors, the integrin receptors, facilitates the second most studied pathway, complement-mediated phagocytosis. Integrins, which are cell adhesion molecules, have the ability to dynamically change their adhesion based on a process called inside-out signaling or priming, allowing binding of ligands to be enhanced as they contact more receptors (Luo *et al.* 2007). CR3, also known as $\alpha_M\beta_2$ integrin or Mac-1, is the most well-known receptor. This receptor is known to have the capability of binding 30 different ligands, being able to bind both opsonized and un-opsonized targets (Le Cabec *et al.* 2002). CR3-mediated phagocytosis requires the GTPases RhoA and Rap1. RhoA in this process recruits mDia formins, a group of Rho family effectors that cause actin to undergo nucleation, which initiate formin-dependent actin polymerization, a process that is regulated by profilin, which is recruited by Rap1 (Kim *et al.* 2012; Romero *et al.* 2004; Barry *et al.* 1997; Hall *et al.* 2006). RhoA also activates Rho kinase to phosphorylate the light chain of myosin II, helping to force the actin extensions outwards (Olazabal *et al.* 2002). Rap also regulates cofilin, an actin depolymerization protein that serves to regulate the magnitude of the receptor response in CR3-mediated phagocytosis (Freeman *et al.* 2011). Rac1 and Rac2 have also been shown to play a role in this pathway, however their exact role isn't clear (Hall *et al.* 2006). Similar to Fc γ R-mediated phagocytosis, the process that halts actin polymerization hasn't been identified; however, PI3K is once again suggested to regulate this process. At this point phagocytosis proceeds similarly and once again fuses with lysosomes to degrade the contents of the phagosome.

1.4.3.2 Macropinocytosis

The other form of macroscale endocytosis, and the one most relevant to this study is macropinocytosis. Macropinocytosis is a highly regulated and conserved mechanism for the bulk, non-selective uptake of extracellular fluid. First discovered in 1931,

macropinosomes were first observed to be large ($>0.2\ \mu\text{m}$) vesicles produced by sheet-like extensions of the plasma membrane, which are called ruffles (Kerr *et al.* 2009). Unlike phagocytosis, macropinocytosis is thought to occurring spontaneously or by stimulation by growth factor receptors. Macropinocytosis begins with ruffles formed at the plasma membrane, which usually retract back into the cell; however, some may fold back and fuse with the membrane enveloping extracellular media, forming macropinosomes. The macropinosomes eventually fuse with lysosomes as they mature, or recycle back to the plasma membrane (Kerr *et al.* 2009). Macropinocytosis has major functions in antigen presentation and pathogen entry, and may be important in cell motility (Lim *et al.* 2011). In neutrophils, macropinocytosis has been shown to be important for complement receptor-1 (CR1) internalization. CR1 is an example of a number of receptors important to the chemotactic response in neutrophils (Carpentier *et al.* 1991).

Macropinosomes exhibit many characteristics similar to vesicles in the endosomal/lysosomal and phagolysosomal systems. In macrophages, macropinosomes have been found to be able to acquire makers of late endosomes, such as Rab7, before finally fusing with lysosomes (Racoosin *et al.* 1993). It has been found that sorting nexin 5 (SNX5) is also recruited to newly formed macropinosomes before fusing with lysosomes (Lim *et al.* 2008; Kerr *et al.* 2006).

Like the formation of phagocytic cups, membrane ruffle growth is generated through the protrusion of actin extensions from the membrane, facilitated by actin reorganization and polymerization at the plasma membrane, so proteins that regulate actin dynamics are highly important to macropinocytosis. The Ras superfamily of proteins has a number of members which are necessary for the formation of the macropinosomes, such as Ras, RhoG, and most importantly, Arf6 and the Rho GTPases, Rac1, Cdc42, and RhoA (Swanson 2008). Many of these proteins will be outlined in further detail below, however, their main purpose in actin reorganization is to activate the WASp and WAVE complexes. As previously discussed, these complexes bind to Arp2/3, as well as PIP2, an important signaling molecule in multiple forms of endocytosis that is generated from the phosphorylation of phosphatidylinositol 4-phosphate (PI(4)P) by phosphatidylinositol 4-

phosphate 5-kinase (PIP5K). This double binding activates Arp2/3 initiating actin nucleation through the direct assembly of an actin monomer onto an existing actin filament (Kerr *et al.* 2009). The proteins p21-activated kinase-1 (Pak1) and CtBP1/BARS are also highly involved, with their purpose being closure and scission of the macropinosome (Liberali *et al.* 2008; Edwards *et al.* 1999). Figure 3 summarizes some of the pathways involved in macropinocytosis above which will be further discussed below.

1.5 Macropinocytic Regulation

1.5.1 ADP-ribosylation Factor 6

ADP-ribosylation Factor 6 (Arf6) is a small GTPase that localizes to the plasma membrane and the endosomal compartments, and is part of the Arf family of GTPases affecting vesicular trafficking and actin reorganization. As a GTPase, Arf6 cycles through an inactive GDP-bound form, and an active GTP-bound form. Function-wise, Arf6 has been implicated in many processes depending upon where recruitment and activation occurs. At the plasma membrane, Arf6 has roles in the regulation of both clathrin-dependent and clathrin-independent forms of endocytosis, as well as in cell remodeling (D'Souza-Schorey *et al.* 2006). Arf6 may also be necessary in recycling endosomes for movement back to the plasma membrane (D'Souza-Schorey *et al.* 1998). The major downstream effectors of Arf6 that function in the regulation of actin reorganization are Rac, PIP5K, and phospholipase D (PLD) (Donaldson 2003).

One of the earliest studies connecting Arf6 and macropinocytosis was conducted by Honda *et al.* (1999). In this study, using activity assays against PIP5K, which generates PIP2, they were able to observe PIP5K activation by Arf6, as well as a number of other Arfs. Furthermore, they found that activation of PIP5K required phosphatidic acid (PA), which is generated by PLD. In order to verify which Arf was physiologically responsible

for PIP5K activation, they transfected C-terminal haemagglutinin (HA) epitope-tagged Arfs and N-terminal myc epitope-tagged PIP5K in HeLa cells. What they found was that both Arf6 and PIP5K localized on the plasma membrane, more specifically in ruffles being formed at the membrane. They also found that PLD2 translocates to the plasma membrane, corroborating their earlier result that PIP5K activation required PLD activity. Furthermore, membrane ruffling was inhibited through the use of an Arf6 dominant negative mutant. As further validation for the requirement of Arf6-activation of PLD for macropinocytosis, a study carried out by O’Luanaigh *et al* (2002) found that PLD2 and Arf6 activity together were required for membrane ruffling in mast cells. Another study conducted the same year by Radhakrishna *et al* (1999) indicated that Arf6 activity regulated the activity of Rac1, another protein important for actin reorganization and macropinocytosis. In this study, they found that Arf6 and Rac1 colocalized to both the plasma membrane and recycling endosomes in HeLa cells and primary human fibroblasts, further verified through colocalization of transfected cells. Furthermore, their role in macropinocytosis was suggested through the observation that cells expressing wild-type Rac1 and Arf6 began to form ruffles after application of the G-protein-coupled receptor agonist, bombesin, and ruffle formation was inhibited through by an Arf6 dominant negative mutant. Since then many more lines of evidence have appeared to validate the role of Arf6 regulation in actin remodeling through PLD, PIP5K, and Rac1 (Brown *et al.* 2001; Hernández-Deviez *et al.* 2004; Boshans *et al.* 2000). Therefore, it is apparent that Arf6 plays an important role in the regulation of macropinocytosis, especially at the initial stages of forming membrane ruffles.

1.5.2 The Rho GTPases

In the regulation of actin dynamics and generation of membrane ruffles. Arf6 has been suggested to be an upstream effector of the Rho family of GTPases, Rac1, Cdc42, and RhoA (Osami *et al.* 2010, Donaldson 2003, Boshans *et al.* 2000). The direct connection between Arf6 and these GTPases is unknown, however at least one study suggests that

Arf6 may recruit Kalirin5, a Rho GTPase family GEF, to activate these proteins downstream (Koo *et al.* 2007).

1.5.2.1 Rac1

The small GTPase, Rac1, is part of the subfamily of Rac proteins, also including Rac2, Rac3, and RhoG (Ridley 2006). All 4 members of the Rac family of proteins localize the plasma membrane, however Rac2 also localizes to the cytosol, while Rac3 and RhoG localize to endosomes. Of the Rac family members, Rac1 has been the best studied. Rac1 is important for the function of many processes, such as lamellipodia formation, membrane ruffling, the neuronal cell death response, neuronal morphogenesis, cell motility, and phagocytosis (Stankiewicz *et al.* 2014). Generally speaking, the function of Rac1 is in the regulation of actin polymerization and cytoskeletal changes. While the role of Rac1 is implicated in the above processes, the exact pathways through which Rac1 regulates them is not clear, but what is commonly known is that Rac1 has been shown to activate the WAVE complex and Pak1 (Eden *et al.* 2002; Steffen *et al.* 2004; Manser *et al.* 1994; Zhang *et al.* 1998).

The WAVE complex is a well-described regulator of actin polymerization through its association with the Arp 2/3 complex when activated, and is thought to be responsible for much of the actin reorganization related activity that occurs from Rac1. The action of WAVE on Arp2/3 is similar to WASp activity on Arp2/3, directly binding to and activating Arp2/3, which goes on to initiate nucleation of actin monomers onto filaments (Kurusu *et al.* 2009). As for Pak1 function, PAK1 has been shown in a number of studies to regulate actin cytoskeleton remodeling, and more importantly requiring Rac activation to do so (Sells *et al.* 1997; Daniels *et al.* 1998; Dharmawardhane *et al.* 2000). This is corroborated by another study demonstrating Pak1 stimulation of macropinocytosis through observation of dextran uptake in the presence of Pak1 mutants. Additionally, they also demonstrated that a Rac1 dominant negative mutant was unable to inhibit this stimulation of macropinocytosis when co-expressed, suggesting that Pak1 stimulation

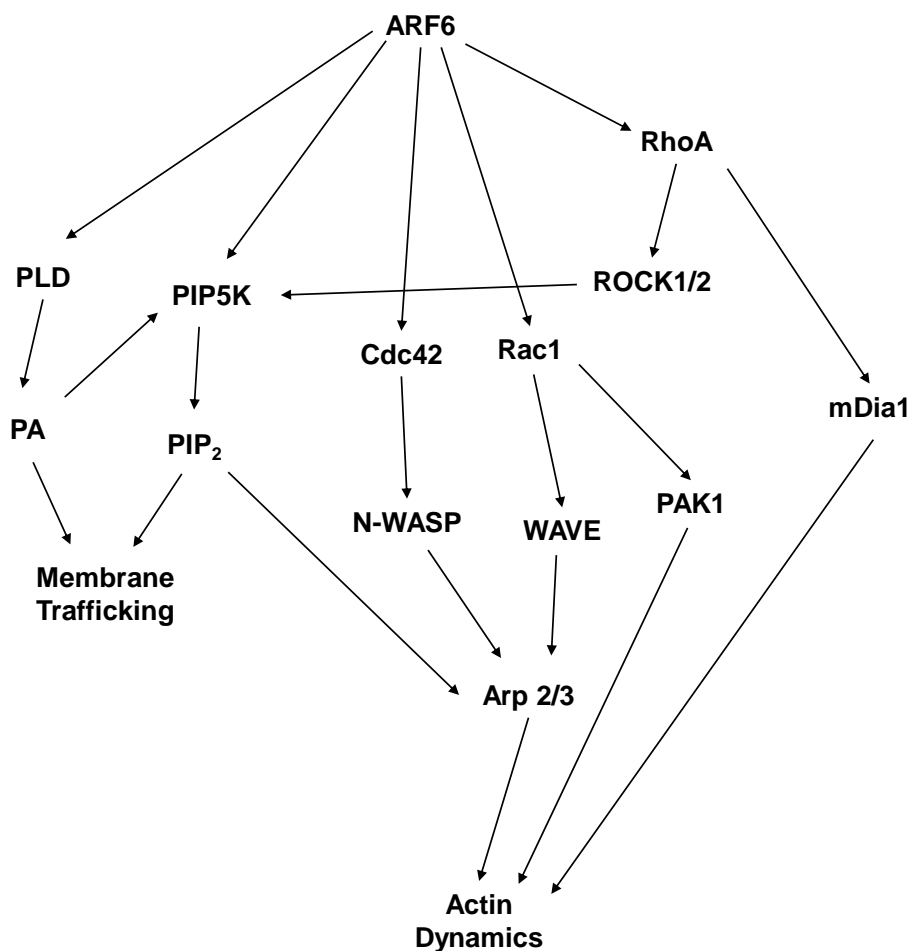


Figure 3. *Regulation of macropinosome formation.* A number of proteins have been implicated in the formation of macropinosomes including the Rho GTPases, PLD, and PIP5K. The diagram above depicts what role they and their downstream effectors have been shown to play in the initiation of macropinocytosis, especially in actin nucleation, as depicted by arrows. The pathway towards actin nucleation begins with Arf6-activation as we believe Arf6 is the major regulator in the macropinocytosis of APP.

downstream of Rac1 may be sufficient for macropinocytosis to occur (Dharmawardhane *et al.* 2000). Pak1 has also been suggested to regulate Arp2/3 activity through the p41-Arc subunit of the Arp2/3 complex (Vadlamudi *et al.* 2004) although the exact mechanism is unknown.

The association between Rac1 and Alzheimer's disease has been noted in multiple studies, however many of these studies report conflicting effects and levels of expression in Alzheimer's disease. A study performed in COS-7 cells showed that Rac1 inhibition was able to decrease γ -secretase activity on APP. Production of the AICD was decreased after Rac1 inhibition, while accumulation of APP CTFs occurred. However, the way this process occurs is a bit unintuitive. What Boo *et al.* (2008) found was that using the inhibitor for Rac1, NSC23766, increased PS1 interaction with Notch, another substrate of γ -secretase. This increased interaction resulted in increased cleavage of Notch by the γ -secretase and decreased cleavage of APP CTFs. Interestingly, another study in primary hippocampal neurons and HEK293 cells showed decreased levels of APP mRNA and protein as a result of Rac1 inhibition using the same inhibitor. This decreased APP mRNA and protein levels were also shown to translate into decreased production of A β 40 and A β 42. Lastly, this study revealed a site within the APP promoter for Rac1 regulation (Wang *et al.* 2009). Rac1 inhibition leading to decreased A β production has also been demonstrated using another Rac1 inhibitor, EHT 1864. Using EHT 1864, Rac1 inhibition was shown decrease γ -cleavage, resulting in increased concentrations of the C99 fragment. Perhaps most relevant to the current study, this inhibition did not reflect a direct effect on γ -secretase activity, as inhibition of Rac1 did not decrease Notch cleavage (Désiré *et al.* 2005). This suggests a possible role for Rac1 inhibition in the trafficking of APP to the intracellular compartment containing γ -secretase activity.

1.5.2.2 Cdc42

The second important Rho GTPase for actin dynamics is Cdc42. Cdc42 localizes to the plasma membrane and the Golgi (Ridley *et al.* 2006). Aside from its role in the regulation

of clathrin-independent endocytosis, Cdc42 is well-known for its role in filopodia and microspike formation, and neuron growth and development (Kozma *et al.* 1995; Ridley *et al.* 1992; Aoki *et al.* 2004; Li *et al.* 2002). However, no matter which process, Cdc42 regulates these actions through stimulation of actin assembly and reorganization. In this aspect, the main activity of Cdc42 is its binding and activation of Wiskott - Aldrich syndrome protein (WASp), which as noted above is able to bind to the Arp2/3 complex to initiate actin nucleation. The association between Cdc42 and WASp has been well documented over the years, and the mechanism of its action has been well described (Aspenström *et al.* 1996; Kolluri *et al.* 1996).

WASp was initially discovered through the identification of its gene in Wiskott-Aldrich syndrome, hence the name (Derry *et al.* 1994). There are two forms of WASp currently known, WASp, and N-WASp, which is highly expressed in neural tissue (Takenawa *et al.* 2007). The role of WASp is to facilitate actin remodeling through the activation of the Arp 2/3 complex, similar to WAVE (Machesky *et al.* 1998). WASp is usually found in a constitutively inactive, folded conformation that covers a site required for Arp2/3 binding. Once bound to PIP2 and Cdc42 the protein becomes activated and unfolds to reveal this domain. Furthermore, binding of either PIP2 or Cdc42 promotes the binding of the other, promoting activation in a co-operative manner (Rohatgi *et al.* 2000). After this occurs, Arp2/3 is stimulated to initiate actin nucleation.

Apart from events regarding actin nucleation, and its necessity for actin reorganization, evidence for a possible role of Cdc42 in the formation of macropinosomes is almost non-existent in the literature. Only one example exists highlighting the possible role of Cdc42 in the regulation of macropinocytosis. In dendritic cells, the ability to produce macropinocytosis is lost as they mature. Garret *et al.* connected this loss in macropinocytic ability to a decrease in the Cdc42-GTPase. In order to test this hypothesis further, they demonstrated that a Cdc42 dominant negative inhibitor for Cdc42 caused ablation of the macropinocytic response in immature dendritic cells. Furthermore, upon delivery of a constitutively active Cdc42 or a Cdc42 GEF revived the capacity for macropinocytosis in mature dendritic cells which lost it (Garrett *et al.* 2000). As for Cdc42 in relation to Alzheimer's disease, there have been few connections demonstrated;

however, one of these is a connection between WASp and Alzheimer's disease. The few studies linking Cdc42 and Alzheimer's disease show that fibrillar A β increases Cdc42 activity, and also that Cdc42 is upregulated in Alzheimer's disease populations (Zhu *et al.* 2000; Moon *et al.* 2013; Mendoza-Naranjo *et al.* 2007). Furthermore, the study from Mendoza-Naranjo *et al.* (2007) suggested that this increase in Cdc42 occurred at the plasma membrane, and resulted in increased actin polymerization activity. As for WASp in Alzheimer's disease, N-WASP protein expression has been found to be increased in Alzheimer's disease brains (Kitamura *et al.* 2003).

1.5.2.3 RhoA

The last Rho GTPase that will be discussed is RhoA. RhoA is part of the Rho subfamily of proteins of which there are three major isoforms: RhoA, RhoB, and RhoC. All three Rho isoforms localize to the plasma membrane, however RhoA and RhoC also localize to the cytosol, while RhoB also localizes the endosome (Ridley 2006). The first and most relevant function of RhoA is in the regulation of actin dynamics. However, RhoA can also upregulate apoptosis, acting antagonistically to Rac1 which suppresses apoptosis. Through this tug-of-war process, Rac1 and RhoA are believed to regulate neuronal cell death and neuron growth (Stankiewicz *et al.* 2014). As evidence for this, Sanno *et al.* found that mice expressing a mutant that causes inhibition of RhoA showed a greater number and density of neurons in the somatosensory cortex, and the overexpression of RhoA in cortical neurons caused an increased rate of apoptosis (Sanno *et al.* 2010). RhoA activates three noteworthy proteins in the regulation of actin reorganization, Rho-associated coiled-coil containing protein kinase (ROCK)-1, ROCKII, and mDia1. mDia1 has been shown to be able to facilitate nucleation of actin (Li *et al.* 2003). In contrast, ROCKI and ROCKII are understood to be inhibitory in actin reorganization, although there are conflicting reports for this. For example, they appear to stimulate apoptosis and inhibit axon growth; however, they have also been shown to stimulate cell proliferation and the formation of stress fibers downstream of RhoA (Julian *et al.* 2014; Fujita *et al.* 2014).

There are very few studies linking RhoA and macropinocytosis, as well as RhoA and Arf6, which will be discussed below. In a study conducted by Zawistowski *et al* (2013) the authors observed the attenuation of RhoA activation in the initial stages of macropinosomes formation. This was then followed by increased RhoA activity after closure of the vesicle. In another study conducted by Kurokawa and Matsuda (2004), RhoA activity was observed to be increased in membrane ruffles and required Cdc42 activity to occur. Suppression of this activity with a dominant negative mutant was also able to override Rac1-stimulated membrane ruffling. The regulation of RhoA by Arf6 has been documented previously in the regulation of actin dynamics, but never further connected to macropinocytosis (Boshans *et al.* 2000; Kim *et al.* 2015).

There is growing evidence that RhoA may be important in Alzheimer's disease. This is best seen with inhibitors against ROCKs, which are downstream of RhoA, and reduce A β production (Zhou *et al.* 2003; Herskowitz *et al.* 2013). In another study, RhoA was found at elevated levels surrounding amyloid plaques in mice expressing the APP Swedish mutation (Petratos *et al.* 2008). In agreement with a connection between RhoA and Alzheimer's disease, Huesa *et al* (2010) indicated that RhoA localizes to synapses and dendritic microtubules in neurons, which changed in APP Swedish mutation mice to become more localized in the neurite and less so in the synapse. Furthermore, in AD patient brains, RhoA was found to localize to neurons, colocalizing with hyperphosphorylated Tau protein.

1.5.3 Phospholipase D

The PLDs are a small family of proteins consisting of PLD1, PLD2, PLD3, PLD4, and PLD5 in humans. Of these proteins, PLD1 and PLD2 are the major isoforms, although PLD3 has been found to have implications in Alzheimer's disease (Cruchaga *et al.* 2014; Satoh *et al.* 2014). PLD1 is localized primarily to the membranes of organelles in the cell, such as endosomes, lysosomes, and the Golgi. PLD2 on the other hand localizes to the plasma membrane. PLD3, which localizes to the endoplasmic reticulum (ER), has also

been seen to be highly expressed in neurons, with Alzheimer's disease brains showing greatly reduced expression (Cruchaga *et al.* 2014; Frohman 2015).

The most commonly studied function of PLD is its capacity to hydrolyze phosphatidylcholine (PC), the most abundant membrane phospholipid, into choline and PA. PA itself is able to produce negative curvature in membranes when accumulated due to a small, negatively charged group on its head; this function may facilitate the generation of vesicles at the membrane. PA also acts as a lipid anchor for a number of guanine nucleotide exchange factors (GEFs) that then activate other proteins such as Rac1 and Ras. As noted above, PA can also activate recruited proteins such as PIP5K (Frohman 2015).

To date, there appear to only be two studies connecting PLD function to macropinocytosis. The first study, conducted by Haga *et al.* (2009), found that PLD1 was required for EGF-stimulated macropinocytosis in Cos7 cells. Furthermore, PLD1-facilitated macropinocytosis required the activation of CtBP1/BARS to occur. The other study, from Mettlen *et al.* (2006) found that ruffling and macropinosome formation was annulled through the use of PLD inhibitors. However, while PA does not directly regulate macropinocytosis, PA does regulate other proteins that have been implicated in actin reorganization, such as PIP5k as mentioned above (Roach *et al.* 2012). PLD and by extension PA, have also been found to be necessary for phagocytosis with PLD1 and PLD2 possibly playing complementary roles, as PLD1 appears in internalized and forming phagosomes, while PLD2 only localizes to forming phagosomes (Corrotte *et al.* 2006; Iyer *et al.* 2004).

1.5.4 PIP5-Kinase

Phosphatidylinositol 4-phosphate 5-kinase (PIP5K) is a phospholipid kinase, as its name suggests, whose main role is the phosphorylation of PI(4)P to produce phosphatidylinositol 4,5-di-phosphate (PI(4,5)P₂) or simply PIP₂. PIP5K activity is

regulated by various upstream hormones, neurotransmitters, and growth factors, in order to temporally and spatially control PIP2 generation within the cell. In mammals there are three distinct isoforms of PIP5K that follow α , β , γ nomenclature. Furthermore, within the PIP5K γ isoform, there exist multiple splice variants. In terms of localization, PIP5K α localizes to the nucleus and cytosol, PIP5K β localizes to the perinuclear region in small punctate structures, and PIP5K γ localizes to focal adhesions, the cytosol, or the nucleus depending on the splicing variant. However, when stimulated, both PIP5K α and PIP5K β quickly localize to the plasma membrane and begin to generate PIP2 (Funakoshi *et al.* 2011).

PIP5K is regulated upstream by a large number of proteins, most of which are activators of PIP5K and important to actin dynamics. The few that are most relevant to this study are Arf6, the Rho GTPases, and PA. AP-2, a regulator of clathrin-mediated endocytosis as mentioned above, also activates PIP5K. Beginning with Arf6, PIP5K activation through Arf6 was first identified by Martin *et al* (1996) in the plasma membranes of HL60 cells where they discovered that the Arf protein could cause overproduction of PIP2 through PIP5K activity. A few years after this, the study by Honda *et al* (1999) discussed previously as well as a study carried out by Jones *et al* (2000) substantiated these results for Arf activation of PIP5K. However, the Honda study showed PIP5K activation by Arf6, while the Jones study demonstrated PIP5K activation by Arf1. One interesting difference in the activation pathways for the two Arf6 proteins on PIP5K was the role of PA. In the Honda study it was noted that PA was required for Arf6-mediated PIP5K activity, indicating a role for PLD in this pathway as well. In contrast, the Jones study showed that PA activity was actually inhibitory, rather than stimulatory. At present, Arf6-activated PIP5K has been shown to regulate a variety of trafficking systems, including membrane ruffling, clathrin-mediated endocytosis, endosomal trafficking, and even some forms of exocytosis. Unfortunately, the function above most relevant to the current study, membrane ruffling, occurs through currently unidentified means.

Other than Arf6, the Rho GTPases Rac1, Cdc42, and RhoA have also been shown to activate PIP5Ks, providing additional links for PIP5K activity in the regulation of actin remodeling (Tolias *et al.* 2000; Chong *et al.* 1994; Weernink *et al.* 2004). However, this

activation, at least for RhoA and Rac1, seems to be indirect as the study by Honda *et al* (1999) looking at a PIP5K activity assay observed that RhoA and Rac1 association with PIP5K was not able to directly activate PIP5K. These findings have been supported, at least for RhoA, by a number of studies that implicate Rho-associated coiled coil-forming protein kinase (ROCK), a downstream effector of RhoA, in the direct activation of PIP5K (Yamazaki *et al.* 2002; Oude Weernink *et al.* 2000). Lastly, activation of PIP5K by PA has been demonstrated in a number of other studies apart from the finding in Honda *et al.* All three of the PIP5K isozymes have been found to be activated by PA (Isihara *et al.* 1998; Jenkins *et al.* 1994; Moritz *et al.* 1992). Unfortunately, whether this activation is directly from PA or through indirect means, as displayed in the Arf6 activation of PIP5K, is currently unknown. As evidence for the role of PIP2, which as discussed above is generated by PIP5K, in macropinocytosis, PIP2 has been shown to increase in concentration at the formation of macropinosomes (Araki *et al.* 2007). Aside from this, PIP2 is well known to play a role in actin rearrangements (Yin *et al.* 2003).

1.6 Rationale and Aims

Our laboratory has discovered evidence for the colocalization of APP, presenilin-1, and LAMP1 in lysosomes, as well as γ -secretase activity in lysosomes, implicating the lysosome as a possible site of APP processing into the toxic A β (Pasternak *et al.* 2003). We have also uncovered a novel pathway for APP processing in which APP is rapidly internalized from the plasma membrane directly to lysosomes, in a pathway completely separate from endocytosis arising from early and late endosomes (Lorenzen *et al.* 2010). Through confocal microscopy of fixed and live cells, and electron microscopy, we were able to see the internalization of APP at the cell surface into large >500 nm vesicles, which then quickly fused with lysosomes (Tang *et al.* 2015). Furthermore, when transfected with Arf6 bearing a dominant negative mutation, this pathway was blocked, while leaving endocytosis of APP into early endosomes intact. By using the Arf6 dominant negative mutant we were also able to show that the observed decrease in lysosomal trafficking of APP was accompanied by a significant reduction in A β

production. Lastly, through immunostaining of human brains, we were able to show an increase in Arf6 expression throughout the hippocampus with the pathogenesis of Alzheimer's disease (Tang *et al.* 2015). With such promising evidence for the role of Arf6 in Alzheimer's disease we set out to explore the pathway through which Arf6 may mediate the macropinocytosis of APP into lysosomes.

Our previous results demonstrate a pivotal role for Arf6 in APP trafficking in lysosomes and in A β production. Furthermore, this mechanism is separate from clathrin-mediated endocytosis and appears to form large (>500 nm) vesicles, consistent with macropinocytosis. Consequently, we speculate that members of the Rho GTPases, Rac1, Cdc42, and RhoA, may play a role downstream of Arf6 in this trafficking, due to multiple lines of evidence linking these proteins to macropinocytosis, actin dynamics regulation, and the ability to be regulated by Arf6. **Therefore, I hypothesize that macropinocytosis of APP to the lysosomes and A β production is regulated by the actin remodeling regulators Rac1, Cdc42, and RhoA downstream of Arf6.** The two principal objectives of this project are: 1) To determine if Rac1, Cdc42, and RhoA can regulate macropinocytosis of APP to the lysosomes. 2) If the Rho GTPases can regulate macropinocytosis, can they can regulate A β production? In this thesis, using inhibitors or siRNA knockdown against Rac1, Cdc42, or RhoA and confocal microscopy, I will demonstrate that macropinocytosis of APP directly to lysosomes can be blocked. Furthermore, using the same inhibitors I will demonstrate that both A β 40 and A β 42 production can be reduced.

Chapter 2: Materials and Methods

2.1 Antibodies and Reagents

Antibodies purchased were: Mouse Anti-HA from Sigma-Aldrich, Rabbit Polyclonal Anti-Cdc42 from Santa Cruz Biotechnology, Rabbit Polyclonal Anti-Rac1 from Santa Cruz Biotechnology, and Rabbit Polyclonal Anti-RhoA from Santa Cruz Biotechnology. SN56 cells were obtained from Dr. Jane Rylett. Neuro-2a (N2A) cells were purchased from ATCC. Fluorescently-labeled secondary antibodies and Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit were purchased from Life Technologies (California). EHT 1864, ML 141, SR 3677 inhibitors and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. A β 40 and A β 42 ELISA assay kits were purchased from Life Technologies (California). Mini-PROTEAN TGX Precast Gels were purchased from Bio-Rad. Dulbecco's modified Eagle's medium (DMEM), minimum essential media (MEM), fetal bovine serum (FBS), heat inactivated fetal bovine serum, Hank's balanced salt solution (HBSS), penicillin, streptomycin, and trypsin-EDTA were all purchased from Gibco.

2.2 DNA Constructs

The β APP construct used was generated by Dr. Pieter Anborgh, and was previously demonstrated to traffic and behave in the same way as wild-type APP (Lorenzen *et al.* 2010). A cDNA encoding APP 750-YFP, a gift from Dr. Bradley Hyman, was used to generate this cDNA. First, the signal sequence encoding the N-terminal 17 amino acid signal sequence of APP and the L-E residues required for signal peptide cleavage (Lichtenthaler *et al.* 1999) was cloned using the primers 5'GCTAGCATGCTGCCCCGGTTTG3' and

5'ACGCGTAGCGTAATCTGGAACATCGTATGGGTACTCCAGCGCCCGA3', adding a 3' haemagglutinin (HA) tag, a 5'NheI site, and a 3'MluI site. In order to then generate the shortened β APP construct, the C-terminal 112 amino acids that begin 12 amino acids upstream of the β -cleavage site was cloned using the primers 3'ACGCGTTTCCTGAACTGCTGCCCCGGCTGCTGCATGGAGCCC5' and 3'ATCAAGACGGAGGAGATCTCTG5'. In addition to the shortened construct, these primers also add a 3'MluI site and a 5'SalI site. The first and second products were then ligated into pEYFP-N1 or pECFP-N1 vectors (Clontech). The β APP construct was generated to reduce the possibility of APP being cleaved by a non-secretase enzyme. Constructs similar to β APP have demonstrated the ability to undergo both β - and γ -cleavage (Grimm *et al.* 2008). Furthermore, β APP has been previously demonstrated to have the same intracellular distribution as well as lysosomal trafficking as that of full length APP (Lorenzen *et al.* 2010).

Expression constructs for regulatory proteins bearing dominant negative mutations were YFP-RhoA-T19N, GFP-Rac1-T17N, and eGFP-Cdc42-T17N, all generous gifts from Dr. Susan Meakin. These dominant negative mutants function by abolishing the protein's affinity for GTP, causing them to tightly bind GEFs and further preventing them from activating the normal endogenous GTPases (Wong *et al.* 2006). Dominant negative constructs for these proteins bearing fluorescent fusion proteins have been shown previously to be functional (Kurokawa *et al.* 2005). Regulatory proteins bearing constitutively activating mutations were YFP-RhoA-Q63L, GFP-Rac1-Q61L, and YFP-Cdc42-G12V, all generous gifts from Dr. Susan Meakin. The constitutively active mutations of these proteins causes decreased intrinsic GTPase activity of the mutant proteins, mimicking a constantly GTP-bound state (Hope *et al.* 2008, Longenecker *et al.* 2003, Johnson 1999). Constitutively active constructs for these proteins bearing fluorescent fusion proteins have also been shown previously to be functional (Kurokawa *et al.* 2005, Yoshizaki *et al.* 2003, Itoh *et al.* 2002). LAMP1-YFP was a generous gift from Dr. Walter Mothes and recloned into mCherryFP. Rab5-mRFP was a generous gift from Dr. Stephen Ferguson.

2.3 Cell Culture and Transfection

SN56 neuroblastoma cells were grown in DMEM, supplemented with 10 % (v/v) heat inactivated FBS (Gibco), and 100 µg/ml penicillin/streptomycin (Gibco). Cells were kept in 25 cm² or 75 cm² flasks (Falcon) in an incubator at 37 °C in a humidified atmosphere containing 5 % CO₂ and were split every 3-4 days. Cells were seeded at a density of 3 x 10⁵ cells/35-mm dish (MatTek) one day prior to transfection date. Cells were then transiently transfected with Lipofectamine 2000 according to manufacturer's instructions (Invitrogen) in serum free medium. Following an incubation period of 24 h, cells were differentiated with the addition of 1mM dibutyryl cyclic AMP (dbcAMP; Sigma) to serum free medium (Hammond *et al.* 1986).

Neuro-2a neuroblastoma cells were grown in MEM, respectively supplemented with 10 % (v/v) FBS (Gibco), and 100 µg/ml penicillin/streptomycin (Gibco). Cells were kept in 25 cm² or 75 cm² flasks (Falcon) in an incubator at 37 °C in a humidified atmosphere containing 5 % CO₂ and were split every 3-4 days. Cells were seeded at a density of 4 x 10⁵ cells/35-mm dish (MatTek) one day prior to transfection date. Cells were then transiently transfected with TurboFect according to manufacturer's instructions (Life Technologies) in serum free medium. Following an incubation period of 24 h, cells were differentiated with serum withdrawal in MEM.

2.4 Inhibitor Treatments

Following differentiation, cells received fresh serum-free medium at indicated concentrations of EHT 1864, ML 141, or SR 3677 dissolved in DMSO. 0.1 % DMSO (v/v) serum-free medium was used as a vehicle control. Cells incubated with ML 141 were incubated for 1 hour at 37 °C and 5 % CO₂ before being subjected to internalization experiments. Cells incubated with SR 3677 were incubated for 6 hours at 37 °C and 5 % CO₂ before being subjected to internalization experiments. Cells incubated with EHT

1864 were incubated for 18 hours at 37 °C and 5 % CO₂ before being subjected to internalization experiments.

2.5 Confocal Microscopy

Imaging was performed on a Zeiss LSM-510 META laser scanning microscope using a Zeiss 63X 1.4 numerical aperture oil immersion lens. The optical section thickness was typically 1 micron. ECFP fluorescence was imaged using 458 nm excitation laser and a 458-480 filter set. EGFP and YFP fluorescence was visualized using a 488 nm excitation laser and a BP 500-550 filter set. mRFP and mCherryFP fluorescence was visualized using a 543 nm excitation laser and a LP 560 filter. AlexaFluor 647 fluorescence was imaged using a 633 nm excitation laser, and a LP 650 filter.

2.6 Antibody Cell Surface Labeling

Anti-HA antibody (Sigma-Aldrich) was labeled with Alexa Fluor 647 using a Zenon Alexa Fluor 647 Mouse IgG₁ Labeling Kit (Life Technologies) according to manufacturer's instructions. All internalization experiments were subject to fixed time-course studies. A freshly prepared conjugate was incubated with cells in DMEM, for SN56 cells, or MEM, for N2A cells, on ice for 30 minutes. The conjugate was removed and the cells were washed twice in HBSS pre-warmed to 37 °C. After washing, warm HBSS was added to the dishes and the cells were incubated at 37 °C and 5 % CO₂ for indicated times. Following incubation, cells were fixed with 4% paraformaldehyde on ice for 15 minutes. Cells chosen for study had to show strong expression of both β APP and compartment marker constructs, through strong fluorescence, in addition to normal morphology. Experiments were replicated 3 or 4 times as indicated for each treatment/construct, with 15 cells sampled at each time point.

2.7 A β 40 and A β 42 ELISA

N2A cells were plated at a density of 5×10^5 cells into each well of a 6-well plate with 2 mL of MEM, with the addition of 10% FBS (v/v), overnight. The following day cells were transfected with HA- β APP-CFP for 24 hr. For A β 40 ELISAs following transfection, the cells were given 1 mL of fresh serum-free medium in each well and 10 μ M of the indicated inhibitor or 0.1 % DMSO diluted into the medium and then incubated for 24 hours. For A β 42 ELISAs following transfection, the cells were given 1 mL of fresh serum-free medium in each well and 10 μ M of the indicated inhibitor or 0.1 % DMSO diluted into the medium and then incubated for 48 hours. After culture, 500 μ L of medium was collected and assayed using an ultrasensitive A β 40 or A β 42 ELISA kit (Life Technologies) according to the manufacturer's instructions. Experiments were replicated 4 times for A β 40 and 3 times for A β 42 and data normalized against the control. Data was plotted and analyzed using GraphPad Prism 6.0 software and an unpaired t-test with a 95% confidence interval.

2.8 siRNA Knockdown

SN56 or N2A cells were split as described in the cell culturing subsection. Stealth siRNAs (Invitrogen) were purchased for Rac1 (GCCUGCUCAUCAGUUACACGACCAA), and Cdc42 (CCUUUCUUGCUUGUUGGGACCCAAA). Silencer Select siRNA (Life Technologies) was purchased for RhoA (AGCCUUGAUAGUUUAGAAAtt). Cells were transfected with increasing amounts of siRNA as indicated by Lipofectamine 2000 (Life Technologies) manufacturer's instructions and western blotted to demonstrate knockdown. Cell lysates were collected 3 days after transfection and assayed by western blotting with a 1:1000 concentration of Anti-Rac1 (Santa Cruz Biotechnology), Anti-RhoA (Santa Cruz Biotechnology), or Anti-Cdc42 (Santa Cruz Biotechnology) antibodies.

For trafficking studies, cells were transfected with HA- β APP-CFP, LAMP1-mCherryFP, and 10 nM of siRNA Negative Control, as well as 400 nM of Rac1 siRNA, 200 nM Cdc42 siRNA, or 50 nM RhoA siRNA depending on the experiment. Following 48 hours of transfection cells were differentiated for 1 day and then surface labeled with AlexaFluor 488 Zenon-labeled anti-HA antibodies as previously described and allowed to internalize for 15 minutes at 37 °C. Cells were then fixed with 4 % paraformaldehyde. Following fixation cells were imaged using confocal microscopy and colocalization percentage between β APP and LAMP1 channels was measured. Experiments were replicated 3 times and data normalized against the control. Data was plotted and analyzed using GraphPad Prism 6.0 software.

2.9 Protein Extraction and Western Blotting

SN56 or N2A cells were plated on 60 mm dishes (Falcon) at a cell density of 1.5×10^6 per 60 mm plate. The following day, plates were then transfected with the appropriate DNA constructs and siRNA transcripts. Following 2 days of incubation and 1 day of differentiation, cells were washed in cold PBS and lysed with NP40 lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10 % glycerol, 1 % IGEPAL/NP40) for 30 min at 4 °C. Cells were then scraped, sonicated, and centrifuged at 14,000 rpm for 10 minutes at 4 °C to remove insoluble material. Protein quantification of supernatant was performed using a Pierce BCA protein assay kit (Life Technologies) according to manufacturer's instructions. Total cell lysates were separated in a 12% SDS-PAGE gel by electrophoresis at 140V for approximately 1 hour. Protein was then transferred onto polyvinylidene fluoride (PVDF) membranes using semi-dry transfer at 25V for 30 minutes. Immediately after transfer, membranes were stained with Ponceau solution to visualize quality and location of transferred bands.

PVDF membranes were first probed with Rac1 (1:1,000), Cdc42 (1:1000), RhoA (1:1,000), or α -tubulin (1: 10,000) (Sigma) antibodies, then incubated in HRP-mouse

antibodies (1: 10,000) (Sigma), developed using ECL and imaged. Quantification of western blot images was done using Image Lab software.

2.10 Data Quantification and Analysis

Colocalization analysis was performed on confocal optical sections using Imaris 7.0.2 with Imaris Colocalization module (Bitplane). Using Imaris, thresholds were set to select only the brightest 2% of pixels in the HA-tagged β APP channel, and the brightest pixels past a set pixel intensity that demarcated compartment markers, to ensure only lysosomes or endosomes were considered in analysis. Imaris then generated a colocalization percentage by determining the number of pixels (above threshold) of surface labeled APP that are colocalized with the lysosomal or endosomal marker. 10-15 cells were measured from multiple plates for each replication of an experiment. Graphing and statistical analysis was performed using GraphPad Prism 6.0 using one-way ANOVA with Tukey post-test with a 95% confidence interval.

Chapter 3: Results

3.1 Rapid transit of cell surface-labeled APP to the lysosome in N2A cells

In these experiments we adopted the N2A cell line because it has been shown to exhibit neuronal morphology (Tremblay *et al.* 2010). This cell line was derived from a mouse neuroblast that expresses neuronal morphology and used in many lines of neuronal study. Many of the pilot experiments in our lab were previously performed in SN56 cells, a cholinergic hybrid cell line made by fusing septal cholinergic neurons with neuroblastoma cells. The N2A cell line was adopted here because of concerns that the SN56 cell line was senescing due to high passage number. However, some experiments with negative results here were replicated in SN56 cells to determine if there were traits missing in the N2A cells.

We first set out to confirm that our basic internalization/macropinocytosis experiment works in N2A cells. A basic 15-minute internalization of APP was replicated from a previous study (Lorenzen *et al.* 2010). Cells were transfected with HA- β APP-CFP (green) and LAMP1-mCherryFP (red) then later surface labeled with anti-HA conjugated AlexaFluor 647 Zenon antibodies on ice for 30 minutes. Cells were then allowed to internalize at 37 °C for 15 minutes, or kept on ice as a 0-minute internalization time point (Fig. 3). Cells were then fixed and imaged using confocal microscopy and analyzed for colocalization. To do this, we used Imaris software to set thresholds to identify the 2% brightest pixels from each channel and to calculate the percentage of green (APP) pixels that were colocalized with red (LAMP1) pixels. Representative images are shown in Figure 4A. Results confirm that internalization of APP occurs in N2A cells after 15 minutes. N2A cells exhibit a large increase in colocalization of β APP and LAMP1 after a 15-minute internalization ($25.5 \% \pm 0.9$) compared to 0 minutes of internalization (3.5 %

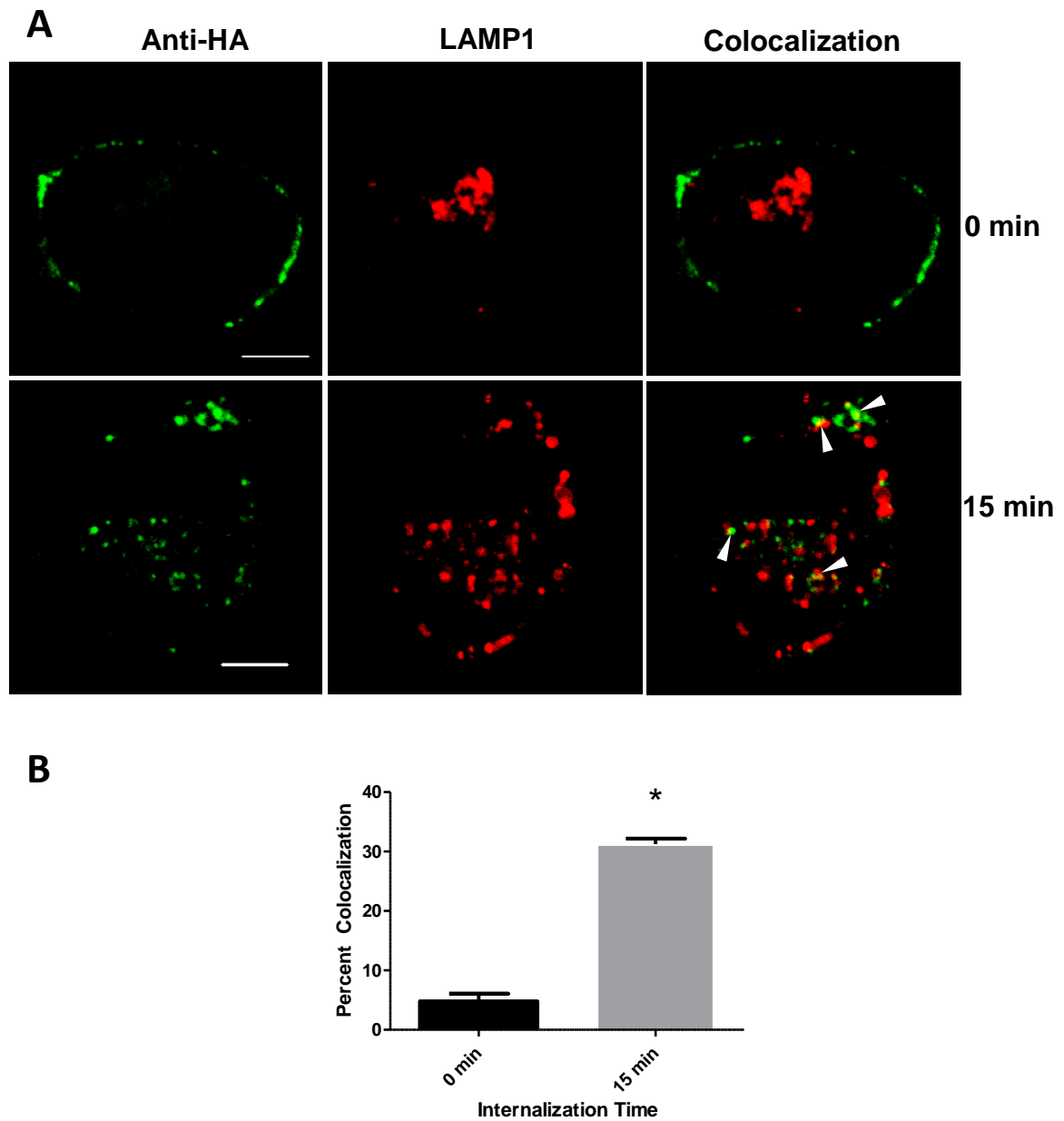


Figure 4. Transport of APP to lysosomes occurs in N2A cells. A) N2A cells transfected with HA- β APP-CFP and Lamp1-chFP (red), surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels for 0 minutes and 15 minutes from 1 experiment. Significance denoted by * ($P < 0.05$) Scale bar = 5 μ m.

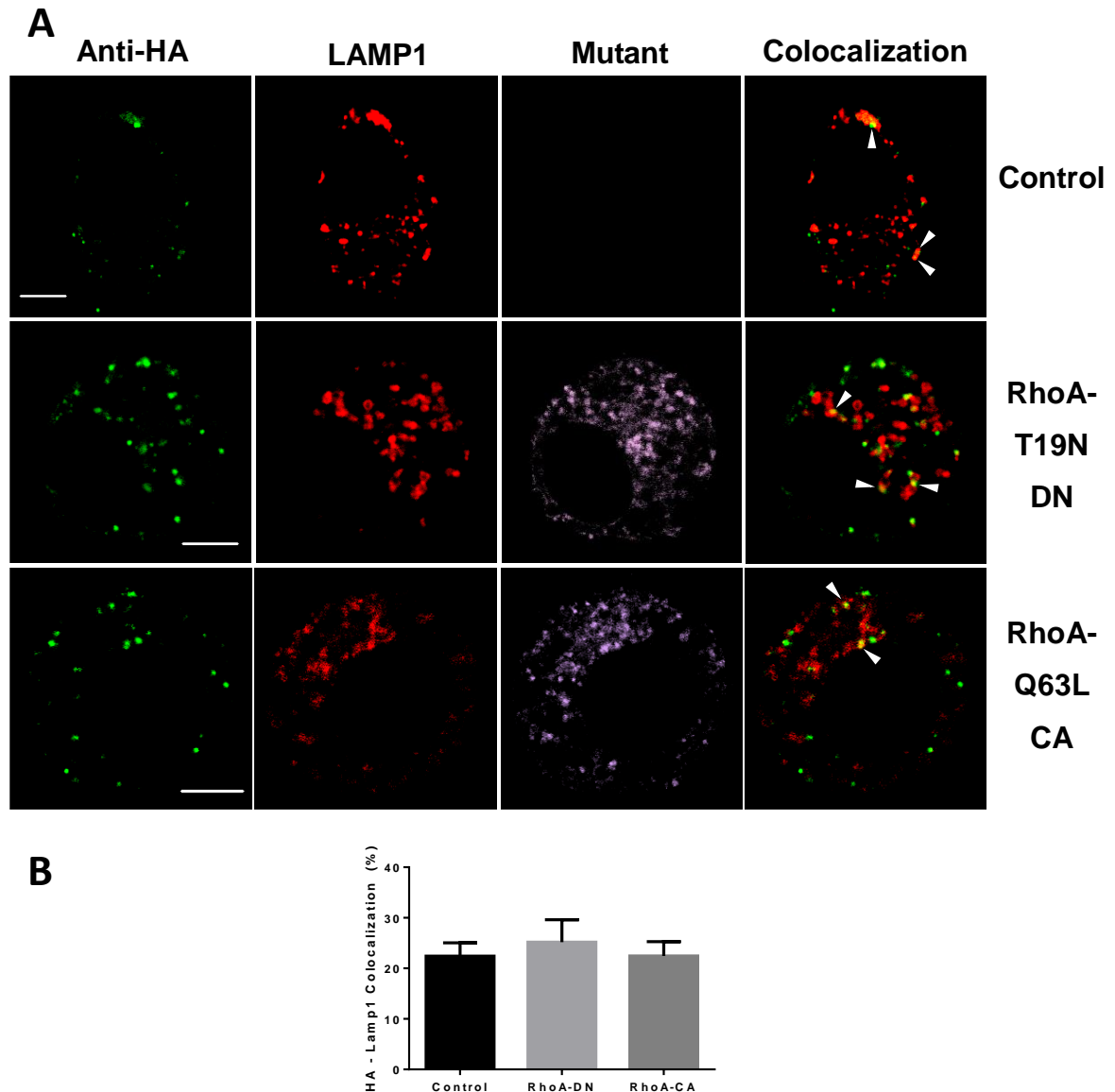


Figure 5. RhoA dominant negative or constitutively active mutants do not affect internalization of APP to lysosomes in SN56 cells. A) SN56 cells transfected with HA- β APP-CFP, Lamp1-chFP (red), and a RhoA dominant negative-YFP or a RhoA constitutively active-YFP mutant, surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for control, RhoA-DN, and RhoA-CA from 5 replicates, no significance between any groups ($p < 0.05$). Scale bar = 5 μ m.

± 1.3). Three replicate experiments, with each experiment examining 10 cells, were analyzed for this experiment.

3.2 Transfection of SN56 cells with RhoA mutants does not affect internalization of APP to lysosomes

RhoA was hypothesized to be downstream of Arf6, because of its effects on actin polymerization. In order to determine if RhoA was able to affect internalization, SN56 cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP a compartment marker for lysosomes, and either RhoA-T19N-YFP, a dominant negative mutant, or RhoA-Q63L-YFP, a constitutively active mutant. After differentiation, cells were surface-labeled with anti-HA conjugated AlexaFluor 647 antibodies, internalized for 15 minutes, fixed, and then imaged with confocal microscopy. Transfection of mutants into cells was observed through fluorescence of the YFP- or GFP-tagged mutants. The colocalization of the brightest 2 % of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 5B). Representative images are shown in Figure 5A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 10 representative cells for each replicate for 5 replicate experiments. There were no significant differences in APP-LAMP1 colocalization between control plates (22.4 % \pm 2.6) and RhoA-DN cells (25.1 % \pm 4.4) or RhoA-CA cells (22.4 % \pm 2.8). Using a one-way ANOVA with a post-hoc Tukey test, for analysis, these differences are not statistically significant. These results would seem to indicate that RhoA does not have an effect on APP trafficking to lysosomes in SN56 cells.

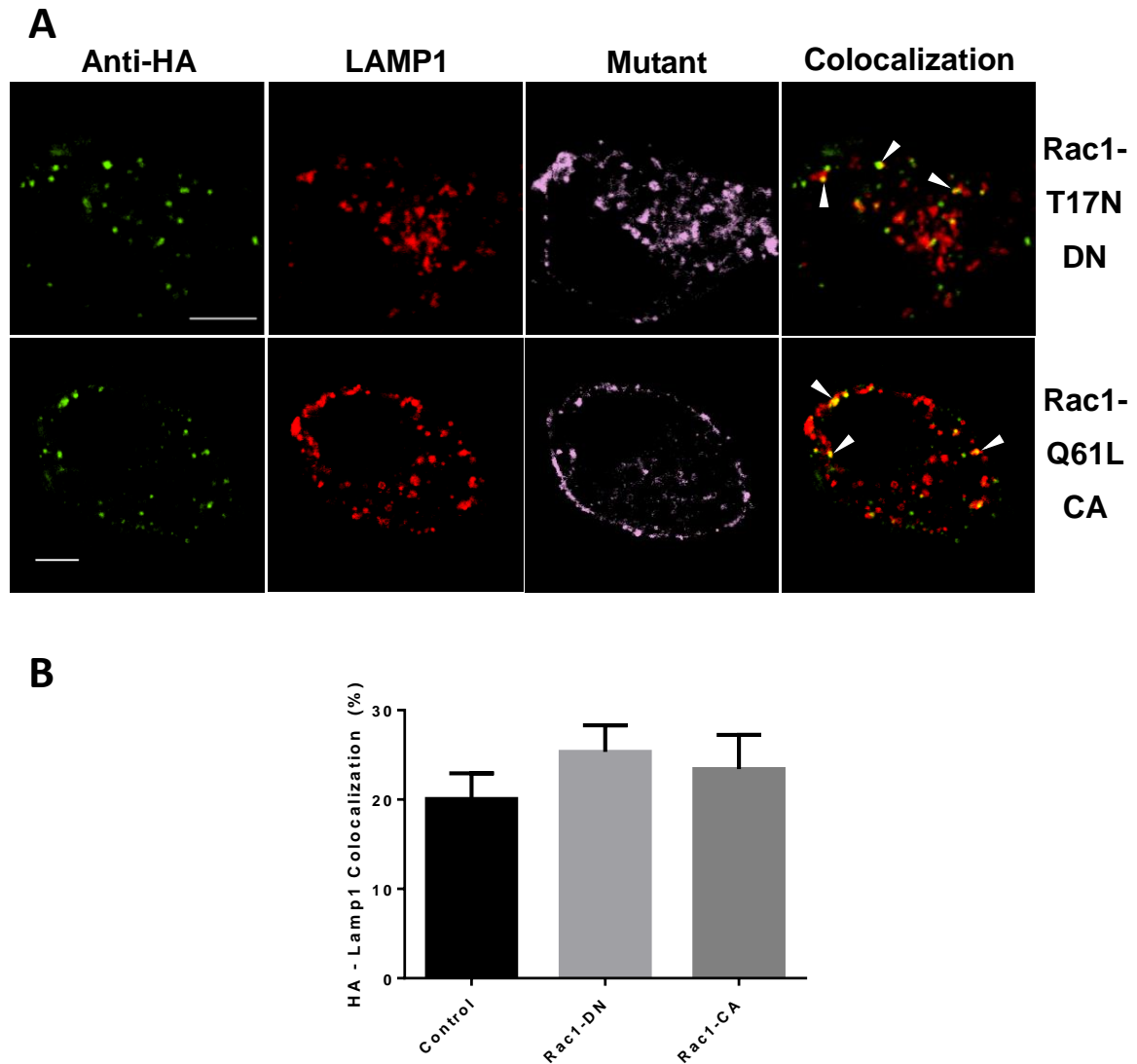


Figure 6. Rac1 dominant negative or constitutively active mutants do not affect APP internalization to lysosomes in SN56 cells. A) SN56 cells transfected with HA-βAPP-CFP, Lamp1-chFP (red), and a Rac1 dominant negative-GFP or a Rac1 constitutively active-GFP mutant, surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA-βAPP and Lamp1 was observed. B) Quantification of colocalization analysis between HA-βAPP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, Rac1-DN, and Rac1-CA from 4 replicates, no significance between any groups ($p < 0.05$). Scale bar = 5 μ m.

3.3 Transfection of SN56 cells with Rac1 mutants does not affect internalization of APP to lysosomes

The next possible mutants downstream of Arf6 to investigate were those for Rac1. Similar to RhoA, Rac1 plays an important role in actin reorganization critical to the formation of macropinosomes and is known to be downstream of Arf6 (Donaldson 2003). To examine the role of Rac1 in the trafficking of APP to lysosomes SN56 cells were used. Cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and either Rac1-T17N-GFP, a dominant negative mutant, or Rac1-Q61L-GFP, a constitutively active mutant. Cells were surface-labeled with anti-HA conjugated AlexaFluor 647 antibodies, internalized for 15 minutes, fixed, and then imaged with confocal microscopy. Transfection of mutants into cells was observed through fluorescence of the GFP-tagged mutants. The co-localization of the brightest 2 % of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 6B). Representative images are shown in Figure 6A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 10 representative cells for each replicate for 4 replicate experiments. Cells in control plates (20.1 % \pm 2.9) were not significantly different from those in cells with Rac1-DN (25.3 % \pm 2.3) or Rac1-CA (23.4 % \pm 3.4) mutants. Using a one-way ANOVA with a post-hoc Tukey test, for analysis, these differences are not statistically significant. These results indicate that transfection of a Rac1 mutant does not affect APP trafficking to lysosomes in SN56 cells.

3.4 Transfection of N2A cells with Rac1 mutants does not affect internalization of APP to lysosomes

Using a newer N2A cell line also exhibiting neuronal morphology we wanted to see if the former negative result with both dominant negative and constitutively active mutants were because of the high passage number of the SN56 cell line. N2A cells were again co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and either Rac1-T17N-GFP, a

dominant negative mutant, or Rac1-Q61L-GFP, a constitutively active mutant. Cells were surface-labeled with anti-HA conjugated AlexaFluor 647 antibodies, internalized for 15 minutes, fixed, and imaged using confocal microscopy. Transfection of mutants into cells was observed through fluorescence of the GFP-tagged mutants. The co-localization of the brightest 2 % of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 7B). Representative images are shown in Figure 7A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 10 representative cells for each replicate for 4 replicate experiments. Cells in control plates ($26.8 \% \pm 3.1$) were not significantly different from those in cells with

Rac1-DN ($34.8 \% \pm 5.2$) or Rac1-CA ($31.7 \% \pm 6.1$) mutants. Using a one-way ANOVA with a post-hoc Tukey test for analysis, these differences are not statistically significant. These results indicate that transfection of a Rac1 mutant does not affect APP trafficking to lysosomes in N2A cells.

3.5 Transfection of SN56 cells with Cdc42 mutants does not affect internalization of APP to lysosomes

To examine the role of Cdc42, SN56 cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and either Cdc42-T17N-GFP, a dominant negative mutant, or Cdc42-G12V-YFP, a constitutively active mutant. Cells were later surface-labeled with anti-HA antibodies conjugated to AlexaFluor 647 secondary antibodies, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. Transfection of mutants into cells was observed through fluorescence of the YFP- or GFP-tagged mutants. The co-localization of the brightest 2 % of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 8B). Representative images are shown in Figure 8A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 10 representative cells for each replicate for 3 replicate experiments. Cells in control plates ($17.9 \% \pm 2.7$) were not significantly different from those in cells with Cdc42-DN ($21.19 \% \pm 2.721$) or Cdc42-

CA ($14.6 \% \pm 1.1$) mutants. Using a one-way ANOVA with a post-hoc Tukey test, for analysis, these differences are not statistically significant. These results indicate that transfection of a Cdc42 mutant does not affect APP trafficking to lysosomes in SN56 cells.

3.6 Transfection of N2A cells with Cdc42 mutants does not affect internalization of APP to lysosomes

The Cdc42 mutants were then examined in N2A cells to observe their effects. Cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and either Cdc42-T17N-GFP, a dominant negative mutant, or Cdc42-G12V-YFP, a constitutively active mutant. Cells were later surface-labeled with anti-HA antibodies conjugated to AlexaFluor 647 secondary antibodies, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. Transfection of mutants into cells was observed through fluorescence of the YFP- or GFP-tagged mutants. The colocalization of the brightest 2 % of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 9B). Representative images are shown in Figure 9A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 10 representative cells for each replicate for 4 replicate experiments. Cells in control plates ($28.9 \% \pm 2.7$) were not significantly different from those in cells with Cdc42-DN ($38.3 \% \pm 2.8$) or Cdc42-CA ($26.9 \% \pm 4.1$) mutants. Using a one-way ANOVA with a post-hoc Tukey test, for analysis, these differences are not statistically significant. These results indicate that transfection of a Cdc42 mutant does not affect APP trafficking to lysosomes in N2A cells.

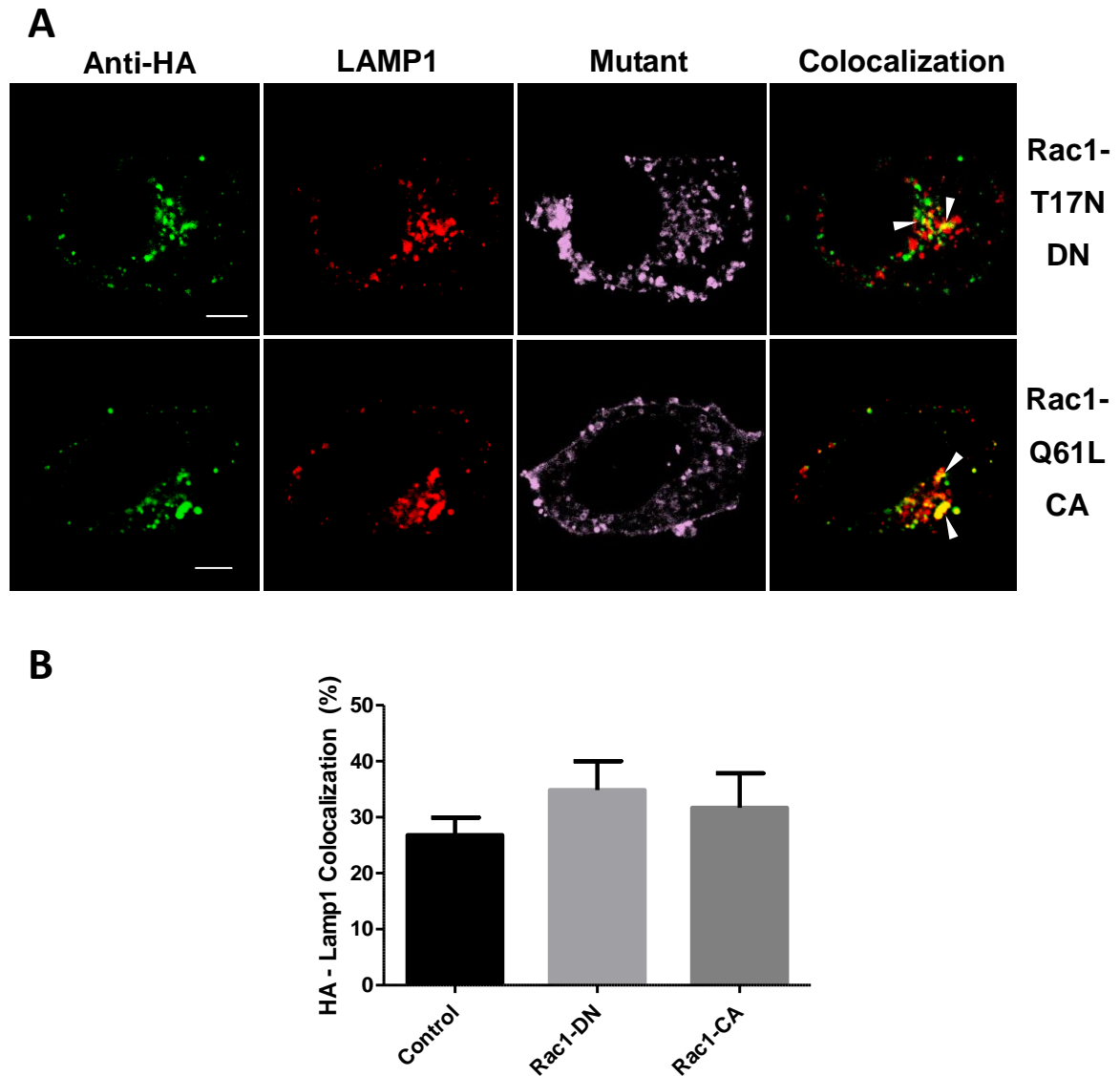


Figure 7. Rac1 mutants do not affect APP internalization to lysosomes in N2A cells.

A) N2A cells transfected with HA- β APP-CFP, Lamp1-chFP (red), and a Rac1 dominant negative-GFP or a Rac1 constitutively active-GFP mutant, surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, Rac1-DN, and Rac1-CA from 4 replicate experiments, no significance between any groups ($p < 0.05$). Scale bar = 5 μ m.

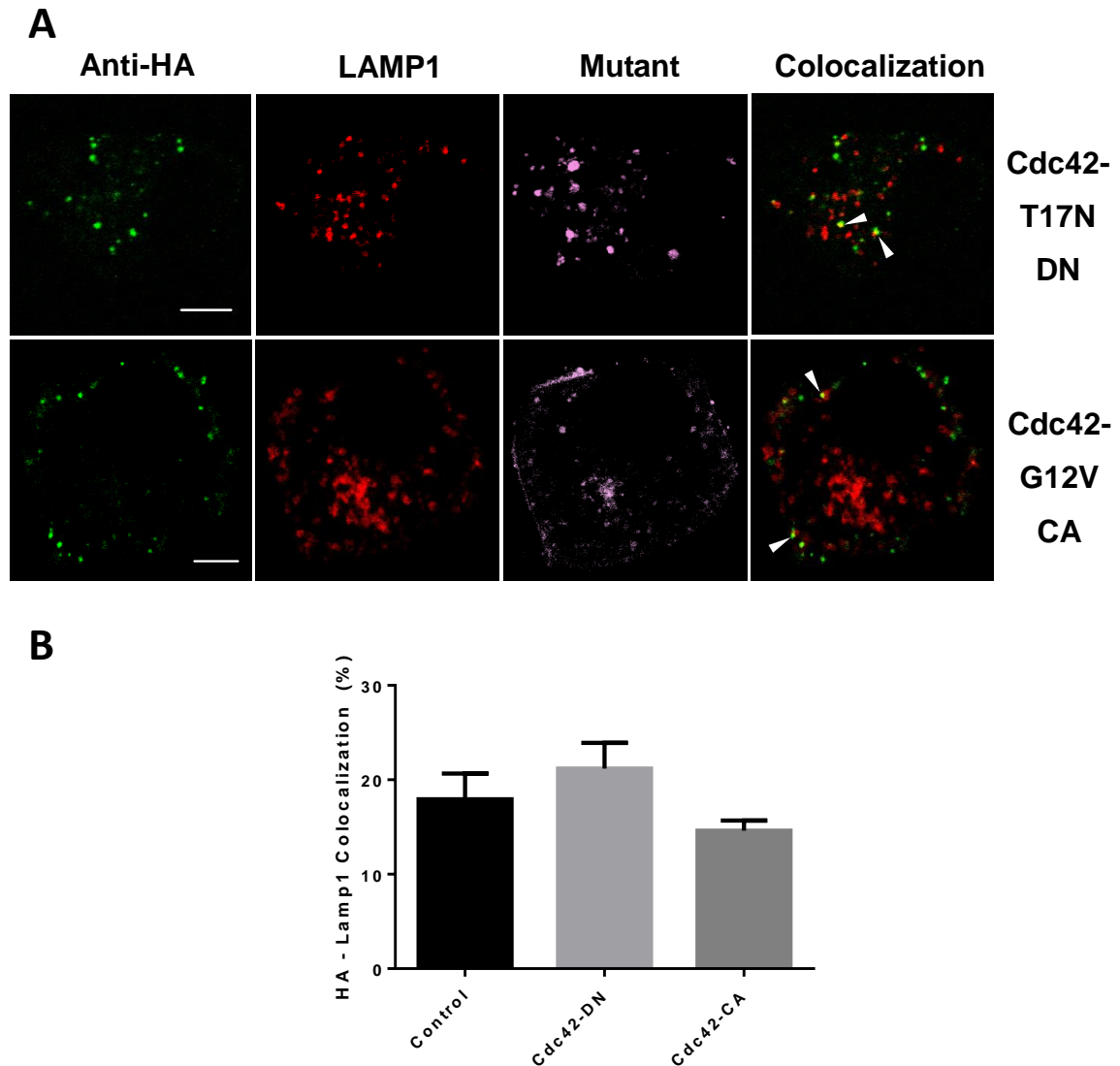


Figure 8. Cdc42 dominant negative and constitutively active mutants do not affect APP internalization to lysosomes in SN56 cells. A) SN56 cells transfected with HA- β APP-CFP, Lamp1-chFP (red), and a Cdc42 dominant negative-eGFP or a Cdc42 constitutively active-YFP mutant, surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, Cdc42-DN, and Cdc42-CA from 3 replicates, no significance between any groups ($p < 0.05$). Scale bar = 5 μ m.

3.7 Inhibition of Rac1, Cdc42, and ROCKII reduces trafficking of APP to lysosomes in a dose-dependent manner

After transfection of Rac1, Cdc42, and RhoA mutants showed no significant differences between groups we wanted to verify these results using another method. To do this an inhibitor for each protein was chosen. The Rac1 inhibitor, EHT 1864, and ROCKII inhibitor, SR 3677, were chosen due to previous studies showing that their usage was able to significantly decrease A β production in cells (Désiré *et al.* 2005; Herskowitz *et al.* 2013).

Furthermore, a ROCKII inhibitor was used because there is currently no commercially available inhibitor for RhoA. The Cdc42 inhibitor (ML 141) is a novel probe not used in many studies, but showing high specificity for Cdc42 GTPase (Surviladze *et al.* 2010). N2A cells were co-transfected with HA- β APP-CFP, and LAMP1-mCherryFP. After transfection and differentiation, cells were incubated in 0.1 % DMSO as a vehicle control for 18 h, EHT 1864 for 18 h, ML 141 for 1 h, SR 3677 for 6 h, or serum-free medium for 18 h. Each incubation was also divided into 3 concentrations to determine dose-dependency based upon concentrations used in previous studies. Incubation times were also based on studies that used them previously (Désiré *et al.* 2005; Herskowitz *et al.* 2013; Hong *et al.* 2013). Afterwards cells were immediately surface-labeled with anti-HA antibodies conjugated to AlexaFluor 647 secondary antibodies, allowed to undergo protein internalization for 15 minutes, fixed, and imaged using confocal microscopy. The co-localization of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes for each inhibitor and concentration (Figure 10B; Figure 11B; Figure 12B). Representative images are shown in Figure 10A for EHT 1864, Figure 11A for ML 141, and Figure 12A for SR 3677. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis.

Inhibition of Rac1 using EHT 1864 caused a significant decrease in trafficking of APP to lysosomes after incubation. Both 10 μ M (14.4 % \pm 2.2) and 20 μ M (14.0 % \pm 1.5)

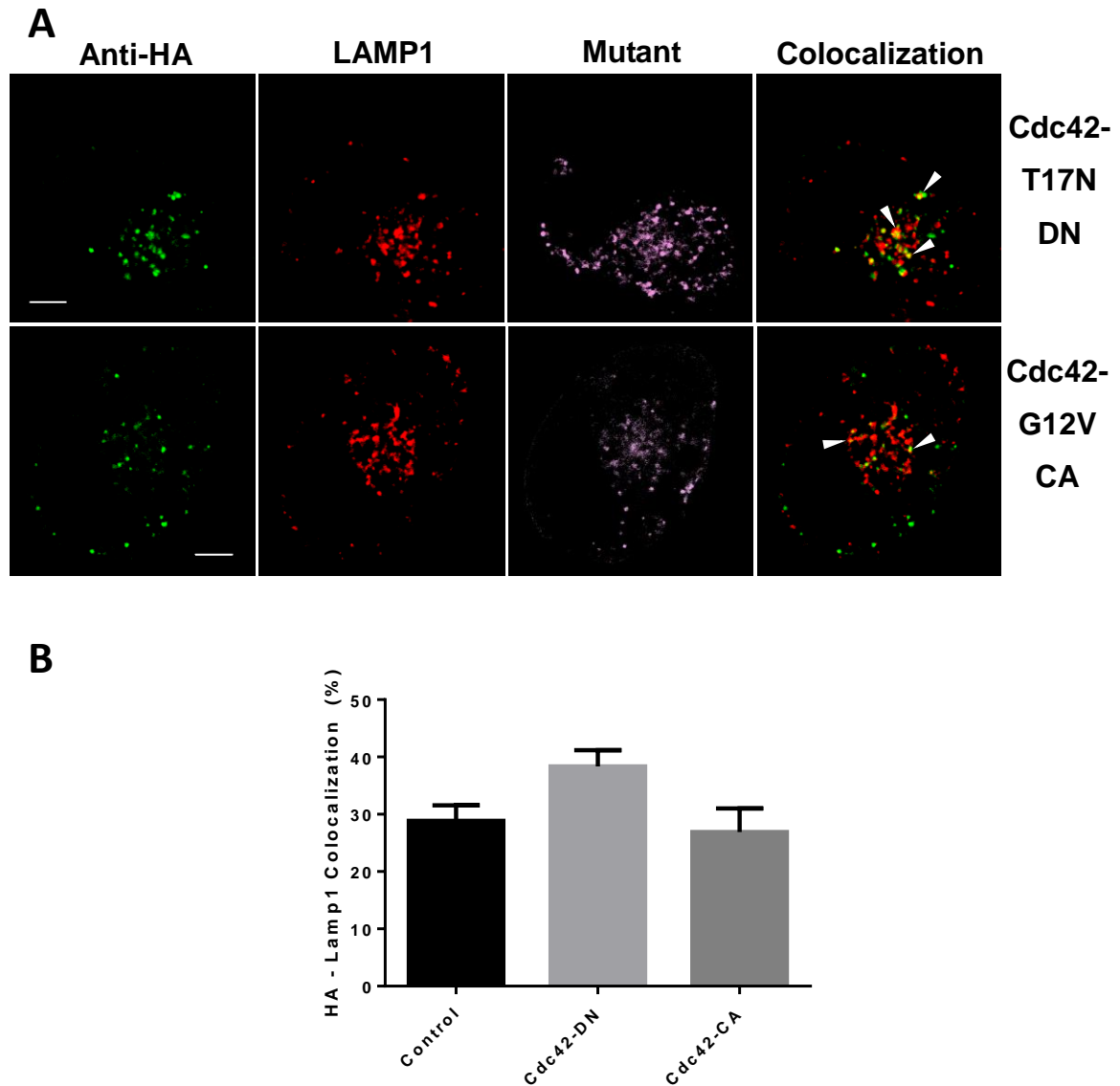


Figure 9. Cdc42 dominant negative and constitutively active mutants do not affect APP internalization to lysosomes in N2A cells. A) N2A cells transfected with HA- β APP-CFP, Lamp1-chFP (red), and a Cdc42 dominant negative-eGFP or a Cdc42 constitutively active-YFP mutant, surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, and then fixed or incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, Cdc42-DN, and Cdc42-CA from 4 replicates, no significance between any groups. Scale bar = 5 μ m.

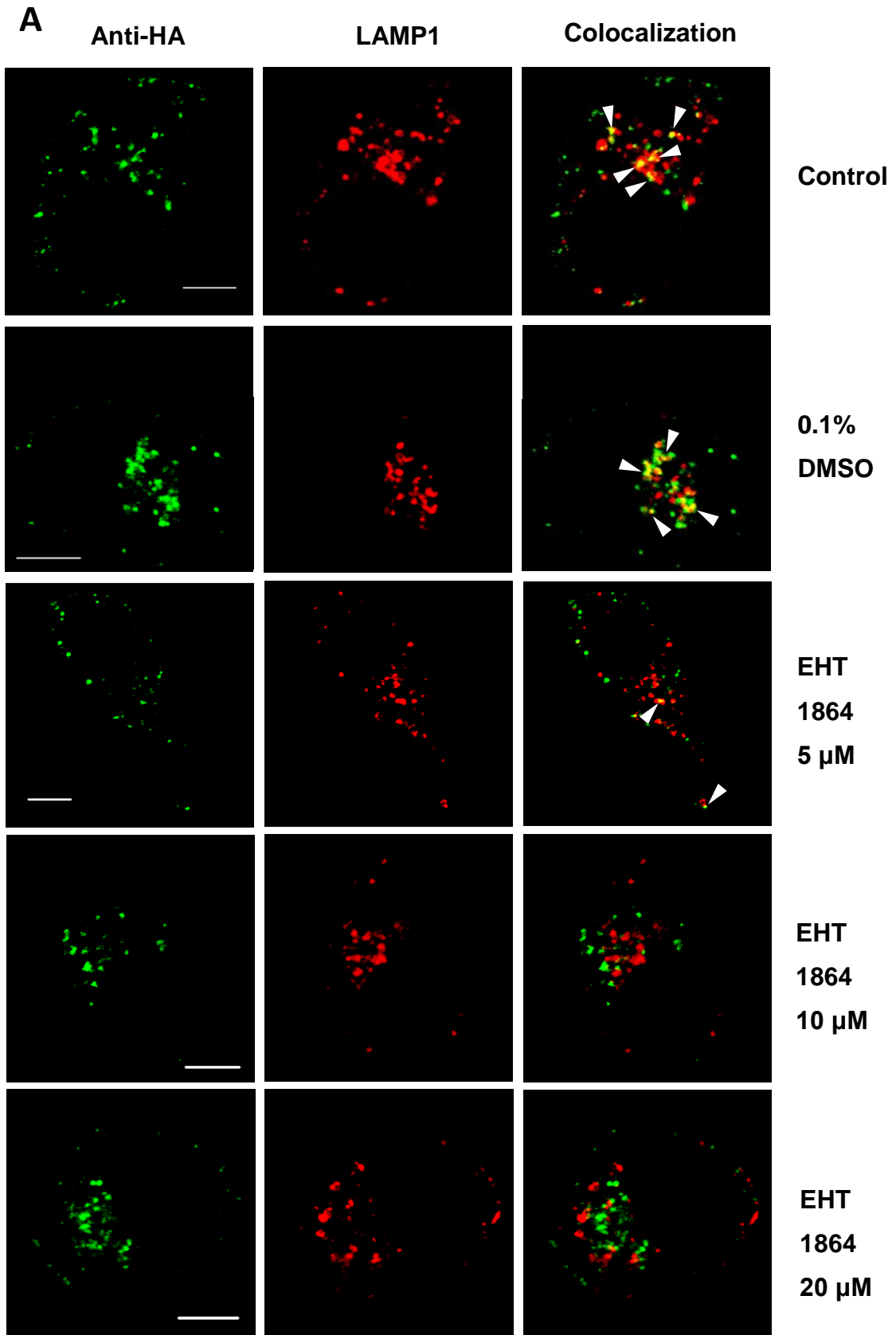
concentrations showed significantly lower colocalization ($p < 0.05$) of APP and lysosomes at 15 minutes compared to control and 0.1 % DMSO cells. The 5 μM concentration ($25.6 \% \pm 2.4$) was not significantly different from either control or 0.1 % DMSO groups, but was significantly higher than 10 μM and 20 μM concentrations ($p < 0.05$). This finding suggests that pharmacological inhibition of Rac1 is able to affect APP trafficking to lysosomes, and in a dose-dependent manner. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments.

Inhibition of Cdc42 using ML 141 also showed a significant decrease in trafficking of APP to lysosomes after incubation. Both 10 μM ($10.3 \% \pm 1.4$) and 20 μM ($7.2 \% \pm 1.4$) concentrations showed significantly lower colocalization of APP and lysosomes at 15 minutes compared to control and 0.1 % DMSO cells ($p < 0.05$). However, the 5 μM concentration ($19.2 \% \pm 5.9$) was not significantly different from either control and 0.1 % DMSO groups, or 10 μM and 20 μM concentrations. This finding suggests that pharmacological inhibition of Cdc42 is able to reduce APP trafficking to lysosomes in a dose-dependent manner. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments.

The last inhibitor used, SR 3677 for ROCKII inhibition, inhibited trafficking of APP to lysosomes after incubation as well. Both 10 μM ($15.6 \% \pm 4.4$) and 25 μM ($11.8 \% \pm 0.3$) concentrations showed significantly lower colocalization of APP and lysosomes compared to 15-minute control and 0.1 % DMSO dishes ($p > 0.05$). Again, the 5 μM concentration ($20.4 \% \pm 1.6$) was not significantly different from either control or 0.1 % DMSO groups, or 10 μM and 20 μM concentrations. This finding suggests that pharmacological inhibition of ROCKII, a downstream effector of RhoA, is able to reduce APP trafficking to lysosomes in a dose-dependent manner. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments.

3.8 Inhibition of Rac1, Cdc42, and ROCKII does not affect trafficking of APP to endosomes

Pharmacological inhibition was able to produce a significant effect on APP trafficking to lysosomes. In order to determine the specificity of this mechanism we examined the effects of Rac1, Cdc42, and ROCKII inhibition on the internalization of APP through classical endocytosis to early endosomes. N2A cells were co-transfected with HA- β APP-CFP, and Rab5-mRFP, a compartment marker for early endosomes. After transfection and differentiation, cells were incubated in 0.1 % DMSO as a vehicle control, EHT 1864, ML 141, SR 3677, or serum-free media for 18 h. For this experiment, inhibitor concentrations were all set at 10 μ M because this was the lowest concentration that showed significant results in each experiment done for lysosomal trafficking, so as to reduce toxicity to the cells. Incubation time was 18 hours for all treatments to allow easier monitoring and internalization of cell cultures; at these incubation times no toxic effects were observed in cells. Cells were then surface-labeled with anti-HA antibodies conjugated to AlexaFluor 647 secondary antibodies, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. The co-localization of pixels from the anti-HA and Rab5 channels was assessed to determine internalization of APP into lysosomes after incubation of each inhibitor (Figure 13B). Representative images are shown in Figure 13A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis. Colocalization analysis results were 21.3 % \pm 3.4 (control), 17.5 % \pm 0.7 (0.1 % DMSO), 19.0 % \pm 2.0 (EHT 1864), 17.3 % \pm 0.8 (ML 141), and 21.6 % \pm 2.1 (SR 3677). None of the groups were significantly different from the others. These results show that inhibition of Rac1, Cdc42, and ROCKII have no effect on APP trafficking through classical endocytosis, while still affecting lysosomal trafficking of APP showing a highly specific way to inhibit this form of APP transport. Furthermore, this data helps to establish a possible pathway for the macropinocytosis of APP downstream of Arf6.



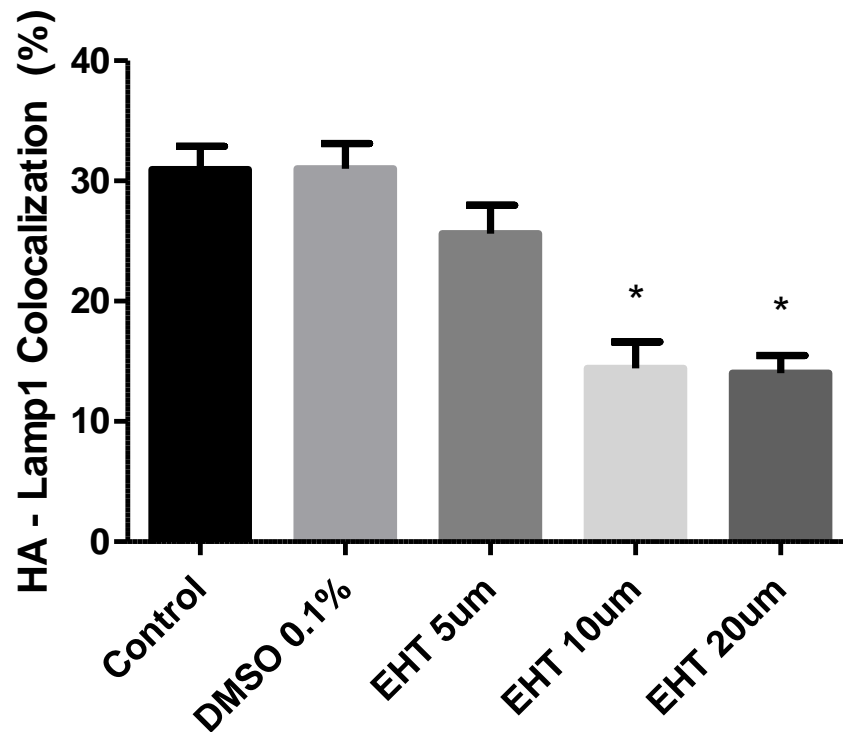
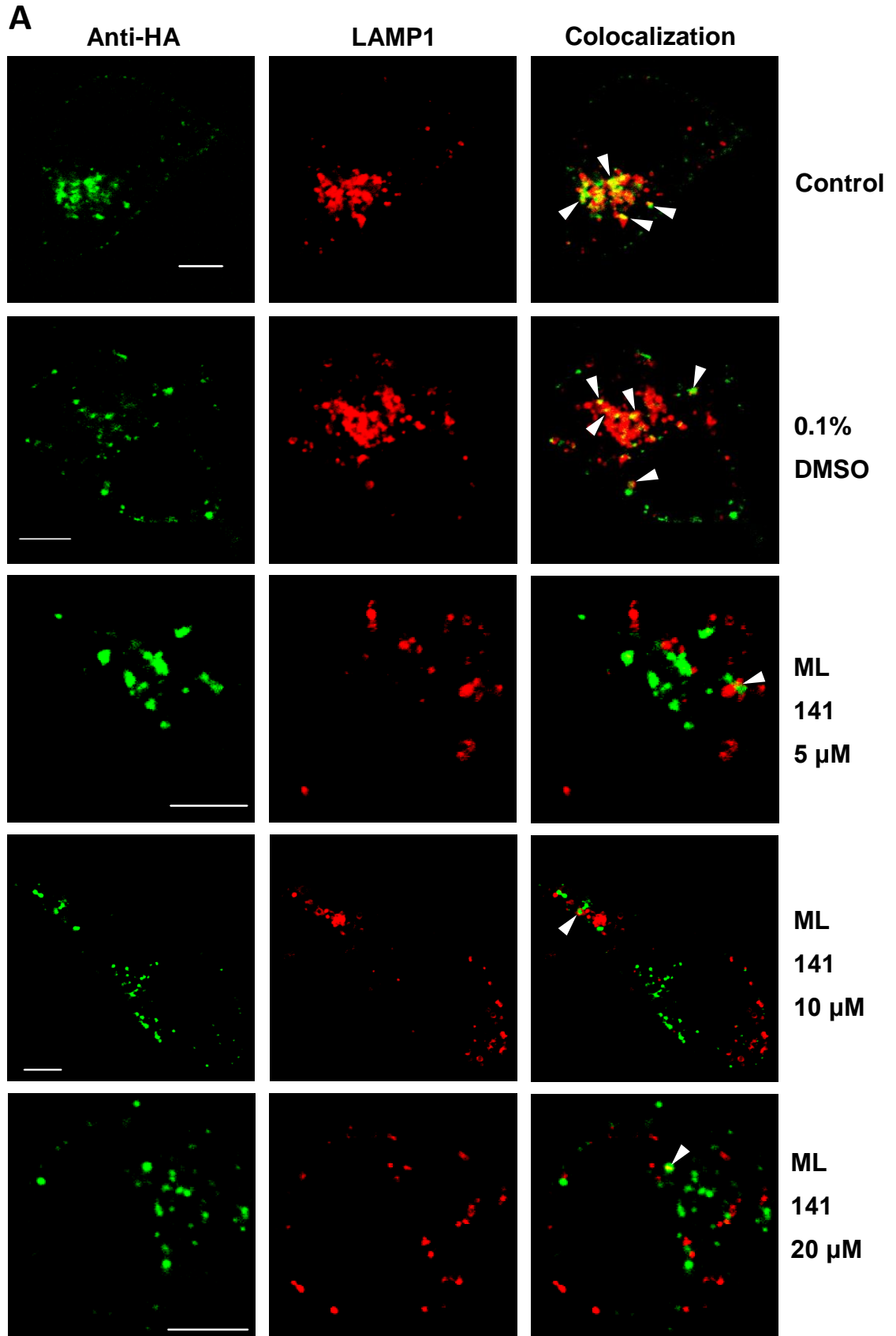
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Figure 10. Internalization of APP is decreased in N2A cells after Rac1 inhibition. A) N2A cells transfected with HA- β APP-CFP (cyan), and Lamp1-chFP (red). Cells were then incubated with EHT 1864 or 0.1% DMSO for 18 hours, and immediately surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, 0.1% DMSO, and EHT using 5uM, 10uM, and 20uM from 3 replicates, significant difference from control is denoted by * ($p < 0.05$). Scale bar = 5 μ m.

3.9 siRNA knockdown of Rac1 in SN56 cells decreases trafficking of APP to lysosomes

A third method was used to examine whether the result from mutant transfection or the result from pharmacological inhibition was what was truly occurring *in vitro*, so an siRNA for Rac1 was chosen. SN56 cells were mock transfected or transfected for 48h with a Negative Control siRNA, or an siRNA against Rac1 for western blotting at 200 nM, 300 nM, and 400 nM concentrations to determine the lowest concentration with a significant effect, to reduce cytotoxic effects. After blotting, protein expression was analyzed after being normalized to α -tubulin expression and expressed relative to the mock transfection (Figure 14C). Data are shown as mean percent colocalization \pm SEM, and quantification used 3 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis. Relative expressions of Rac1 were 1.23 ± 0.05 (Negative Control), 0.86 ± 0.04 (200 nM), 0.94 ± 0.14 (300 nM), and 0.72 ± 0.13 (400 nM). Relative expressions of Rac1 were significantly different between a Negative Control siRNA, which has no homology with any other genes, and 400 nM groups ($p < 0.05$).

For internalization experiments SN56 cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and siRNA against Rac1 at a 400 nM concentrations based upon the previous western blot experiments. A negative control siRNA conjugated to AlexaFluor 647 was also transfected into every plate at 10 nM except for the mock transfection plate in order to visualize which cells were transfected with the negative control. Cells transfected with the negative control were assumed to also be transfected with the Rac1 siRNA, which is not visible. After transfection and differentiation, cells were immediately surface-labeled with anti-HA conjugated to AlexaFluor 488, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. The co-localization of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 14B). Representative images are shown in Figure 14A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 4 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis. Mock ($32.6 \% \pm 0.5$) and



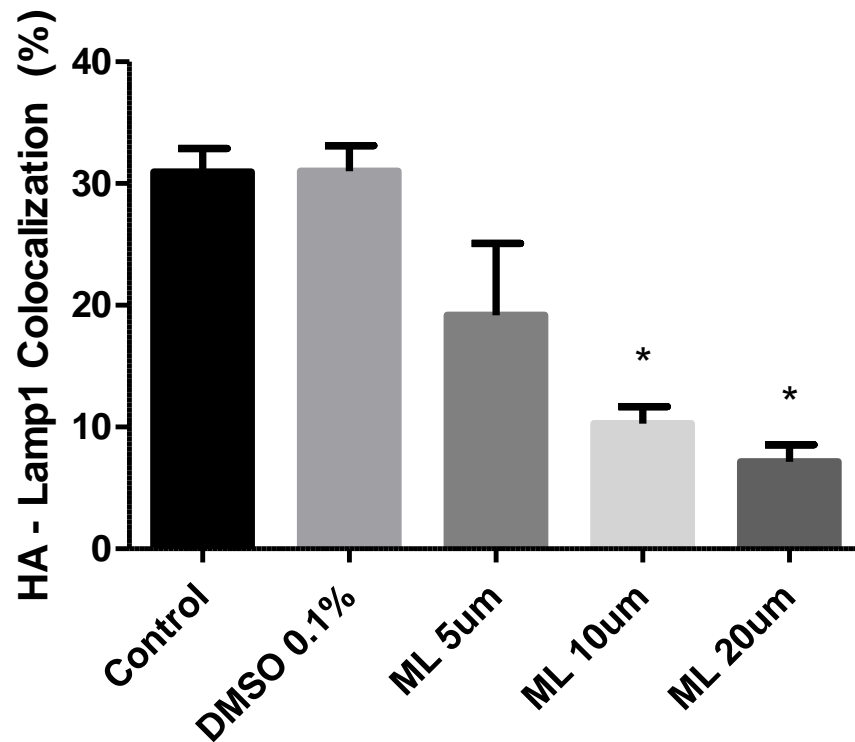
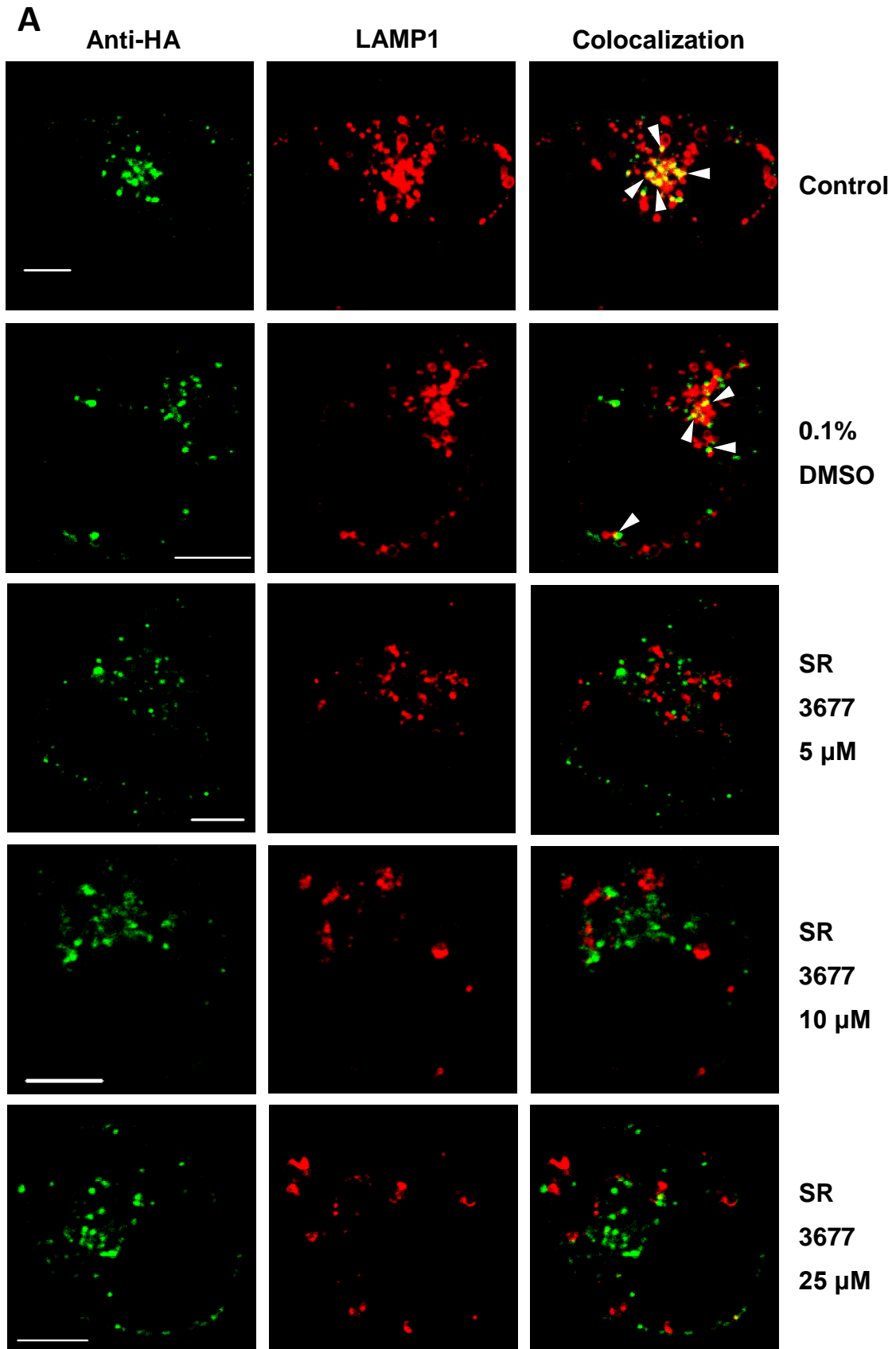
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Figure 11. Internalization in N2A cells is decreased after Cdc42 inhibition. A) N2A cells transfected with HA- β APP-CFP (cyan), and Lamp1-chFP (red). Cells were then incubated with ML 141 or 0.1% DMSO for 1 hour, and immediately surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, 0.1% DMSO, and EHT using 5uM, 10uM, and 20uM from 3 replicates, significant difference from control is denoted by * ($p < 0.05$). Scale bar = 5 μ m.



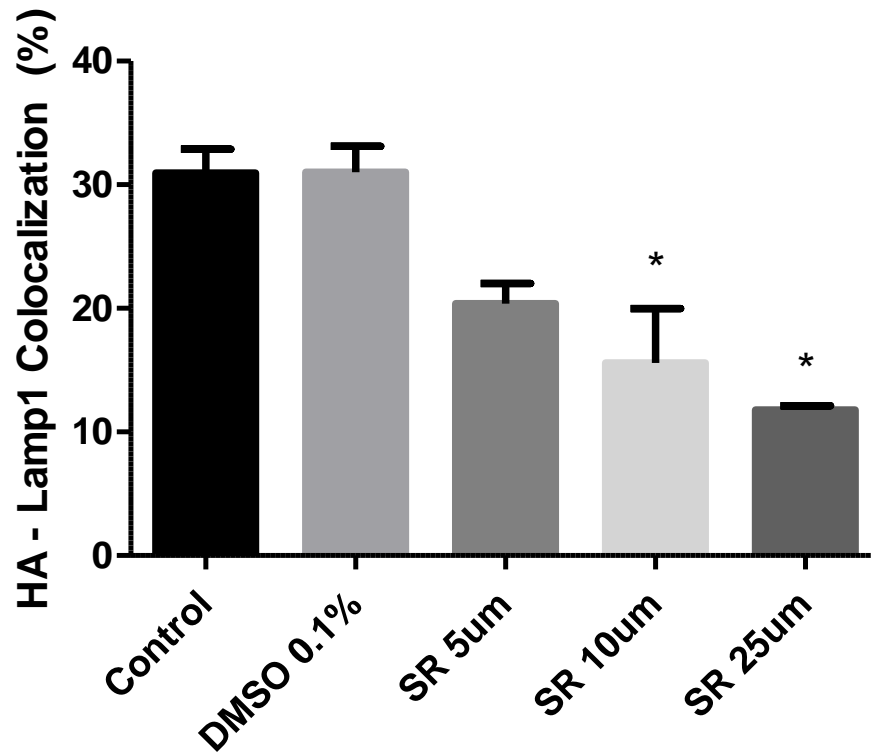
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Figure 12. Internalization in N2A cells is decreased after ROCKII inhibition. A) N2A cells transfected with HA- β APP-CFP (cyan), and Lamp1-chFP (red). Cells were then incubated with SR3677 or 0.1% DMSO for 6 hours, and immediately surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Lamp1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37°C for control, 0.1% DMSO, and EHT using 5uM, 10uM, and 25uM from 3 replicates, significant difference from control is denoted by * ($p < 0.05$). Scale bar = 5 μ m.

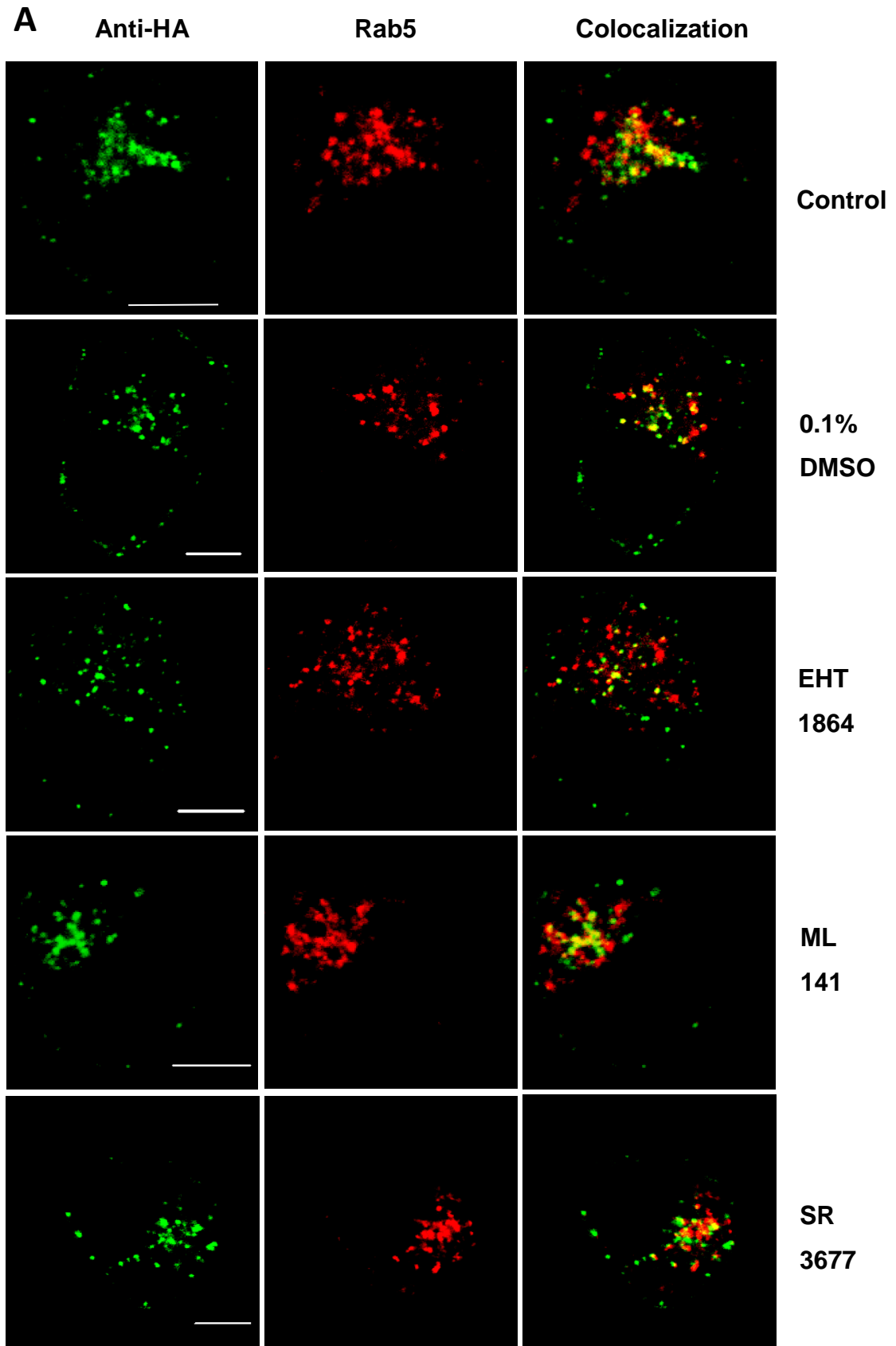
negative control dishes ($30.7 \% \pm 0.5$) showed no significant differences in APP trafficking. However, the 400 nM concentration of Rac1 siRNA ($16.9 \% \pm 3.2$) showed significantly lower ($p < 0.05$) APP colocalized with lysosomes. This suggests that siRNA knockdown through siRNA is able to reduce APP transport to lysosomes.

3.10 Rac1 siRNA was unable to produce knockdown in N2A cells

N2A cells were mock transfected or transfected for 48h with a Negative Control siRNA, or an siRNA against Rac1 for western blotting at 200 nM, 300 nM, and 400 nM concentrations to determine the lowest concentration with a significant effect. After blotting, protein expression was analyzed after being normalized to α -tubulin expression and expressed relative to the mock (Figure 15). Data used 2 replicate experiments. Relative expressions of Rac1 were 1.00 ± 0.09 (Mock), 2.50 ± 1.90 (200 nM), 1.45 ± 0.66 (300 nM), and 1.40 ± 0.47 (400 nM). Using the same Rac1 siRNA as the SN56 cells, a knockdown was unable to be produced in N2A cells. This may have been due to differences in transfection efficiency between the two cell lines, or differences in the levels of Rac1 protein expressed between SN56 and N2A cells. In addition, a single base change in the N2A cells in the region covered by the siRNA could also result in failure of this specific siRNA.

3.11 siRNA knockdown of Cdc42 in N2A cells decreases transport of APP to lysosomes

In order to study the effects of siRNA knockdown on Cdc42, N2A cells were mock transfected or transfected for 48h with a Negative Control siRNA, or an siRNA against Cdc42 for western blotting at 200 nM, 300 nM, and 400 nM concentrations. After



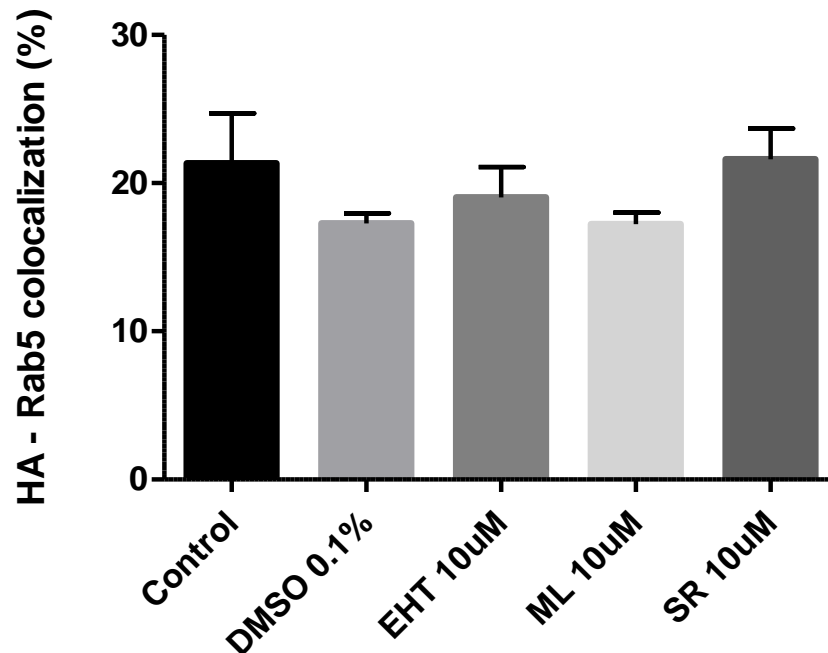
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Figure 13. Internalization of β APP into endosomes is unaffected after inhibition of Rac1, Cdc42, and ROCKII. A) N2A cells transfected with HA- β APP-CFP, and Rab5-mRFP (red). Cells were then incubated with EHT1864, ML141, SR3677, or 0.1% DMSO for 24 hours, and immediately surface-labelled with Zenon-647 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37°C. After 15 minutes of incubation at 37°C, colocalization between HA- β APP and Rab5 was observed. B) Quantification of colocalization analysis between HA- β APP and Rab5 channels with standard error for 15 minute incubations at 37°C for control, 0.1% DMSO, and 10uM EHT1864, ML141, or SR3677 from 3 replicates, no significance between any of the groups ($p < 0.05$). Scale bar = 5 μ m.

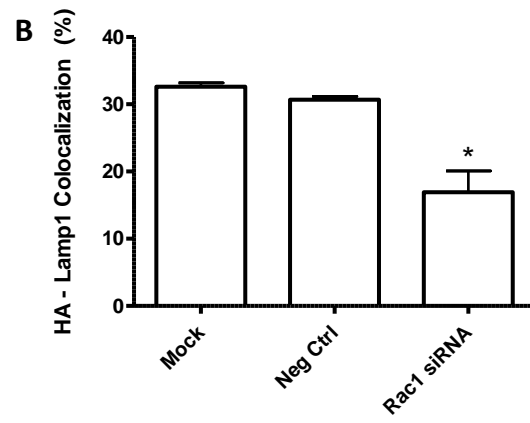
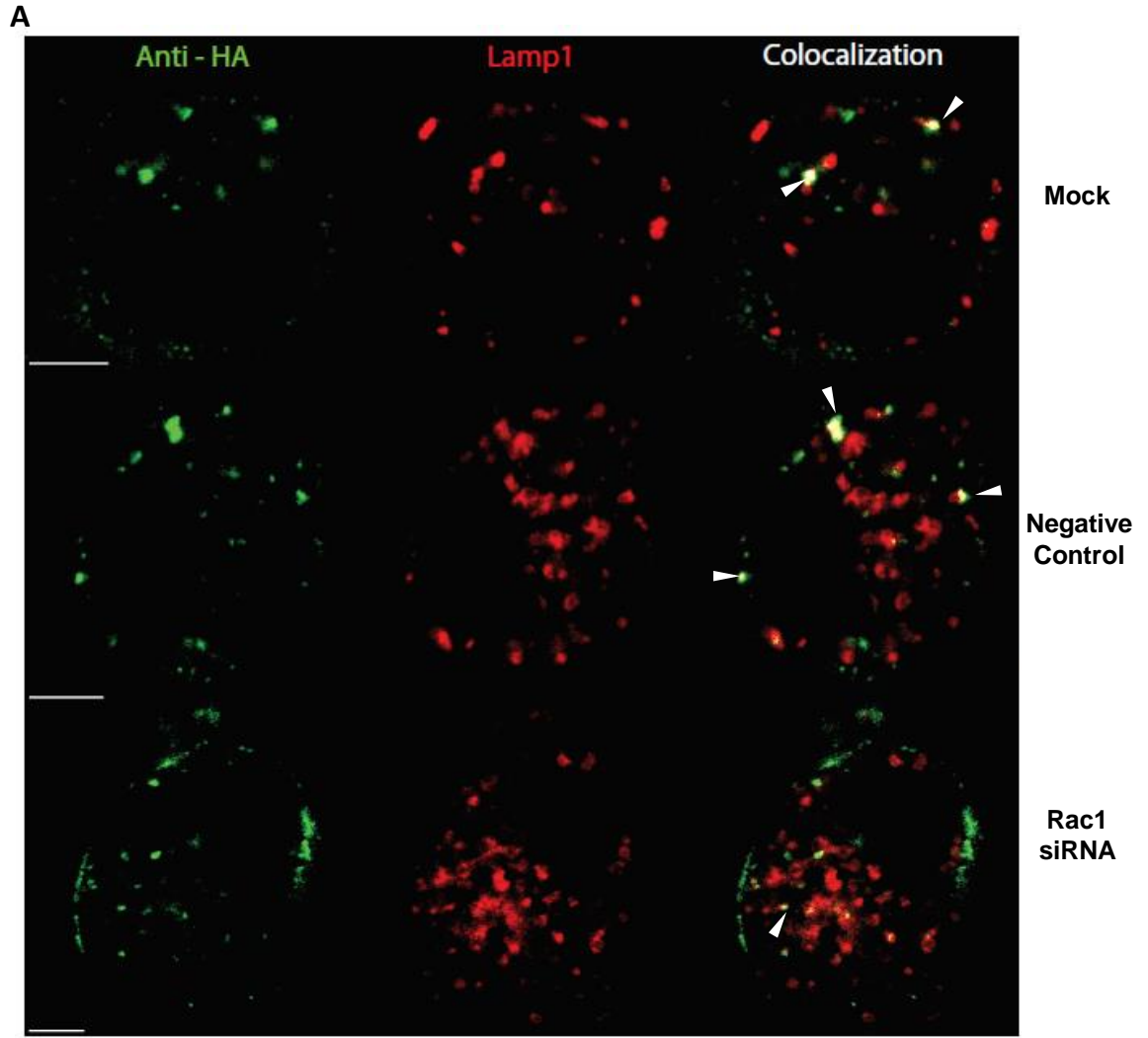
blotting, protein expression was analyzed after being normalized to α -tubulin expression and expressed relative to the mock transfection (Figure 16C). Data are shown as mean percent colocalization \pm SEM, and quantification used 3 replicate experiments, using a two-tailed t-test between each concentration and the mock for analysis. The relative expressions of Cdc42 were 1.01 ± 0.09 (Mock), 0.32 ± 0.19 (200 nM), 0.30 ± 0.25 (300 nM), and 0.24 ± 0.16 (400 nM). The 200 nM and 400 nM concentrations showed significantly lower expression of Cdc42 as compared to the mock transfection ($p < 0.05$).

For internalization, N2A cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and siRNA against Cdc42 at 200 nM based upon the previous western blot experiments. A negative control siRNA conjugated to AlexaFluor 647 was also transfected into every plate at 10 nM except for the mock transfection plate in order to visualize which cells were transfected with the negative control. Cells transfected with the negative control were assumed to also be transfected with the Cdc42 siRNA, which is not visible. After transfection and differentiation, cells were immediately surface-labeled with anti-HA conjugated to AlexaFluor 488, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. The co-localization of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 16B). Representative images are shown in Figure 16A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis. Colocalizations were $28.1 \% \pm 0.7$ (mock), $29.3 \% \pm 3.1$ (negative control) and $6.8 \% \pm 0.7$ (200nM). Cdc42 knockdown with an siRNA displayed significantly lower ($p < 0.05$) APP trafficking to lysosomes. Similar to inhibition of Cdc42 with a pharmacological inhibitor, knockdown of Cdc42 was also able to greatly reduce the transport of APP to lysosomes.

3.12 siRNA knockdown of RhoA in N2A cells decreases APP internalization into lysosomes

The effect of RhoA knockdown was studied using N2A cells and an siRNA against RhoA. For western blotting, N2A cells were mock transfected or transfected for 48h with a Negative Control siRNA, or an siRNA against RhoA at 50 nM, 75 nM, and 100 nM concentrations. After blotting, protein expression was analyzed after being normalized to α -tubulin expression and expressed relative to the mock (Figure 17C). Data are shown as mean percent colocalization \pm SEM, and quantification used 3 replicate experiments, using a two-tailed t-test between each concentration and the mock for analysis. The relative expressions of RhoA were 1.01 ± 0.09 (Mock), 0.31 ± 0.10 (50 nM), 0.47 ± 0.13 (75 nM), and 0.47 ± 0.17 (100 nM). The 50 nM, 75 nM, and 100 nM concentrations all showed significantly lower expression of RhoA as compared to the mock ($p < 0.05$).

For internalization experiments, N2A cells were co-transfected with HA- β APP-CFP, LAMP1-mCherryFP, and siRNA against RhoA at 50 nM based upon the previous western blot experiments. A negative control siRNA conjugated to AlexaFluor 647 was also transfected into every plate at 10 nM except for the mock transfection plate in order to visualize which cells were transfected with the negative control. Cells transfected with the negative control were assumed to also be transfected with the RhoA siRNA, which is not visible. After transfection and differentiation, cells were immediately surface-labeled with anti-HA conjugated to AlexaFluor 647, allowed to internalize for 15 minutes, fixed, and imaged using confocal microscopy. The co-localization of pixels from the anti-HA and LAMP1 channels was assessed to determine internalization of APP into lysosomes (Figure 17B). Representative images are shown in Figure 17A. Data are shown as mean percent colocalization \pm SEM, and quantification used at least 15 representative cells for each replicate for 3 replicate experiments, using a one-way ANOVA and Tukey post-hoc test for analysis. Colocalizations were $28.1 \% \pm 0.7$ (mock), $29.3 \% \pm 3.1$ (negative control) and $9.3 \% \pm 2.1$ (50nM). RhoA knockdown presented significantly lower ($p < 0.05$) APP trafficking to lysosomes. RhoA knockdown, like inhibition previously, was able to significantly reduce the transport of APP to lysosomes.



C

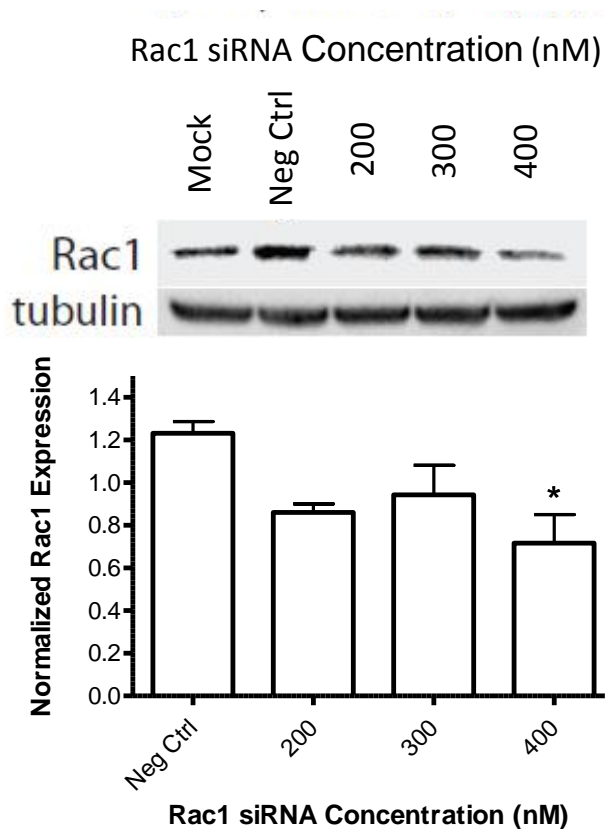


Figure 14. Internalization of β APP into lysosomes is decreased by siRNA knockdown of Rac1. A) SN56 cells transfected with HA- β APP-CFP, Lamp1-chFP (red), a Rac1 siRNA (MSS237709), and Stealth RNAi siRNA Negative Control Med GC-AlexaFluor 647 for 48 hours. Cells were then differentiated for a day and then immediately surface-labelled with Zenon-488 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37 °C. After 15 minutes of incubation at 37 °C, colocalization between HA- β APP and LAMP1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with SEM for 15 minute incubations at 37 °C for mock transfection, negative control, and rac1 siRNA. C) Western blot image and quantification with a mock, negative control, or 200 nM, 300 nM, or 400 nM concentrations of Rac1 siRNA. Data retrieved from 3 replicates for Western Blots and 4 replicates for internalization, significance is denoted by * ($p < 0.05$). Scale bar = 5 μ m.

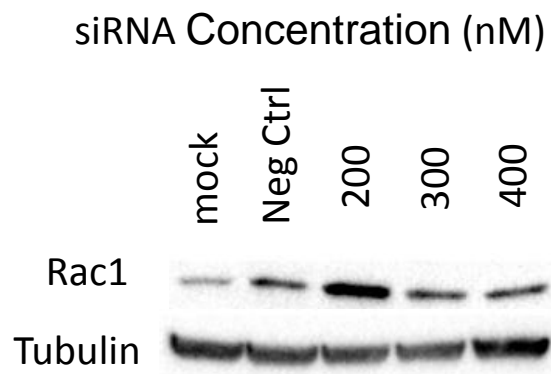


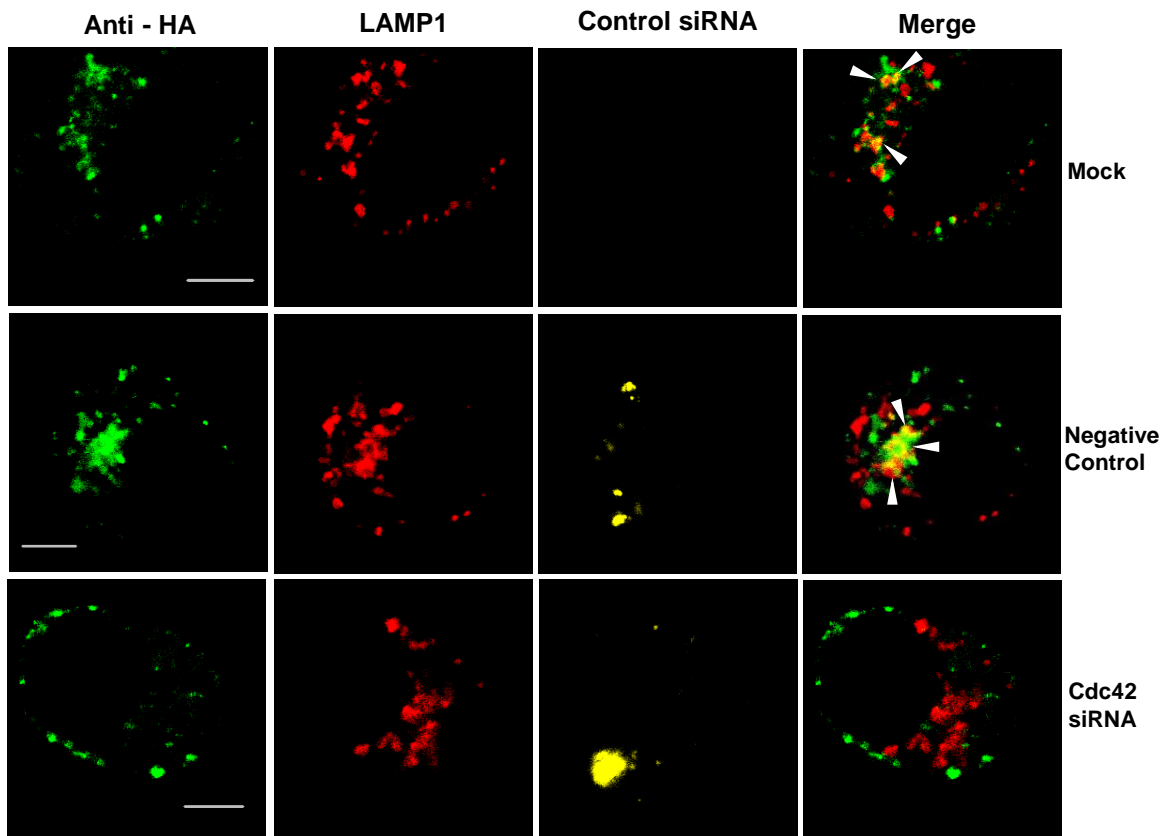
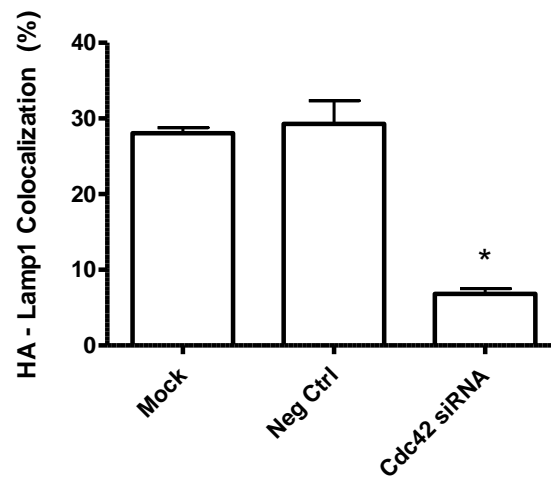
Figure 15. Rac1 siRNA did not induce knockdown in N2A cells. N2A cells transfected with HA- β APP-CFP, Lamp1-chFP (red), a Rac1 siRNA (MSS237709) for 48 hours. Cells were then differentiated for a day before protein extraction. Western blot image from a mock transfection, negative control siRNA, 200 nM, 300 nM, or 400 nM concentrations of Rac1 siRNA. Data is from 2 replicates.

3.13 Pharmacological inhibition of Rac1, Cdc42, and ROCKII show significantly reduced secretion of A β 40

After showing through siRNA knockdown and pharmacological inhibition of Rac1, Cdc42, and RhoA that lysosomal but not endosomal trafficking of APP could be reduced we had to look ahead towards the larger picture. We needed to see whether our observed decreases in APP transport translated to actual decreases in A β production. The first A β peptide studied was A β 40 because A β 40 is generated in larger amounts and we wanted to see whether effects could be observed in its production before moving to A β 42. A Human A β 40 ELISA kit (Invitrogen) was used to analyze cell culture media after pharmacological inhibition. N2A cells were transfected with HA- β APP-CFP and then differentiated. After differentiation cells were incubated with a 0.1 % DMSO vehicle control, or inhibitors at 10 μ M for Rac1 (EHT 1864), Cdc42 (ML 141), or ROCKII (SR 3677) for 24h. This incubation time was chosen to reduce possible cytotoxic effects from incubating for too long while ensuring that there could be enough A β 40 produced to be detectable. After incubation, cell media were extracted and analyzed. Cell media was analyzed in the ELISA as A β is known to be secreted by cells into surrounding fluid, allowing measurement of A β produced (Walsh *et al.* 2002). Experiments were performed with 3 replicates, and data shown represents the mean \pm SEM normalized to the vehicle control, analyzed using a one-tailed t-test. Production of A β 40 after inhibition of Rac1 (56.2 % \pm 6.4), Cdc42 (49.3 % \pm 17.7), and ROCKII (38.3 % \pm 14.8) are all significantly lower than control (110.5 % \pm 20.7) ($p < 0.05$) (Figure 18).

3.14 Pharmacological inhibition of Rac1, Cdc42, and ROCKII also shows reduced secretion of A β 42

The next step after analyzing production of A β 40 was to look at A β 42. This is necessary to study along with A β 40 as A β 42 is the more toxic of the two peptides, and it has been strongly suggested that the ratio between the two may be a key factor in the development

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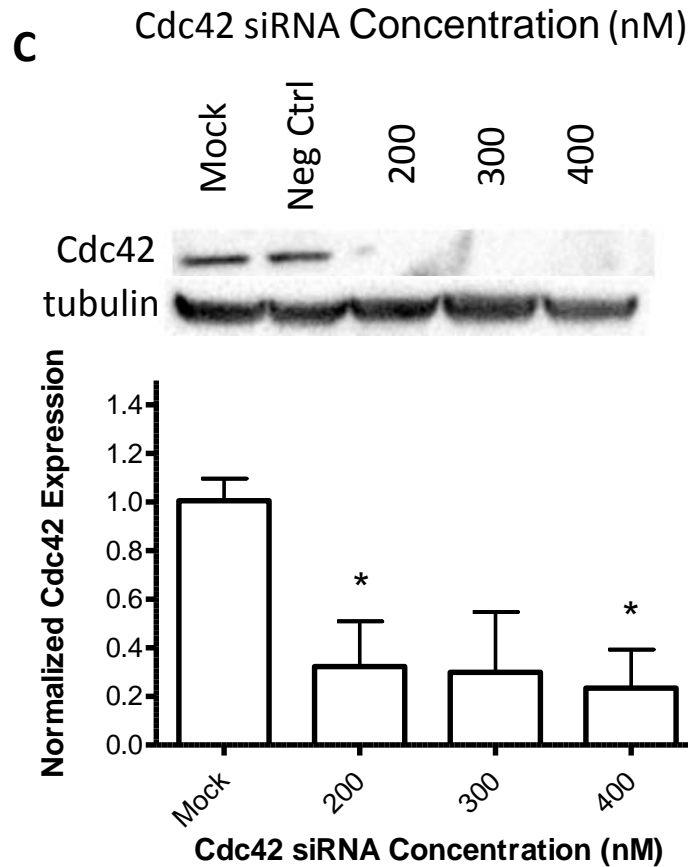
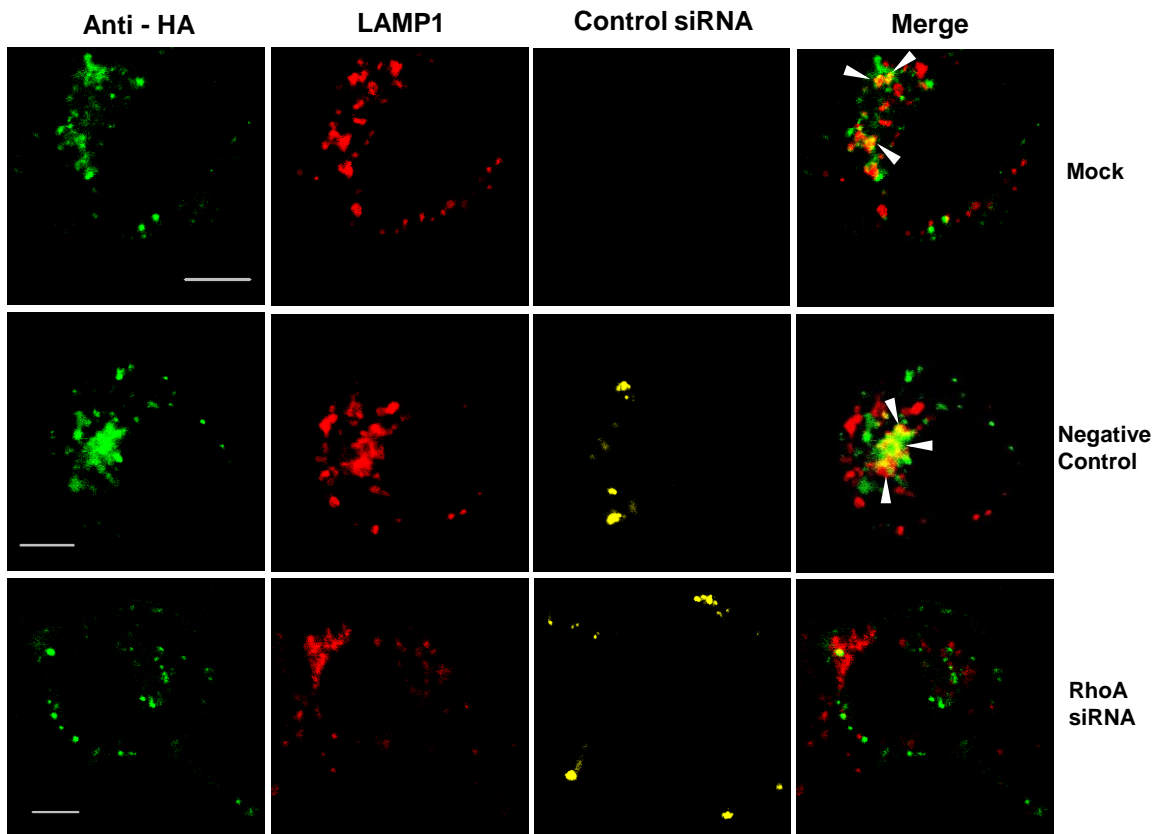
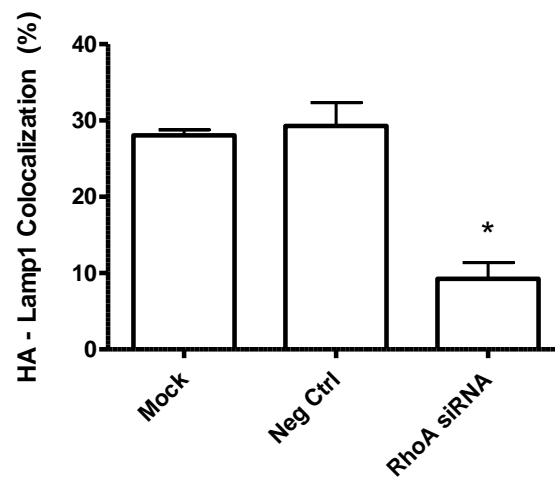


Figure 16. siRNA knockdown of Cdc42 decreases internalization of APP to lysosomes. A) N2A cells transfected with HA- β APP-CFP, Lamp1-chFP (red), a Cdc42 siRNA (MSS247082), and Stealth RNAi siRNA Negative Control Med GC-AlexaFluor 647 for 48 hours. Cells were then differentiated for a day and then immediately surface-labelled with Zenon-488 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37 °C. After 15 minutes of incubation at 37 °C, colocalization between HA- β APP and LAMP1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37 °C for mock transfection, negative control, and Cdc42 siRNA. C) Western blot image and quantification with a mock, negative control, or 200 nM, 300 nM, or 400 nM concentrations of Cdc42 siRNA. Data retrieved from 3 replicates for Western Blots and 3 replicates for internalization, significance is denoted by * ($p < 0.05$). Scale bar = 5 μ m.

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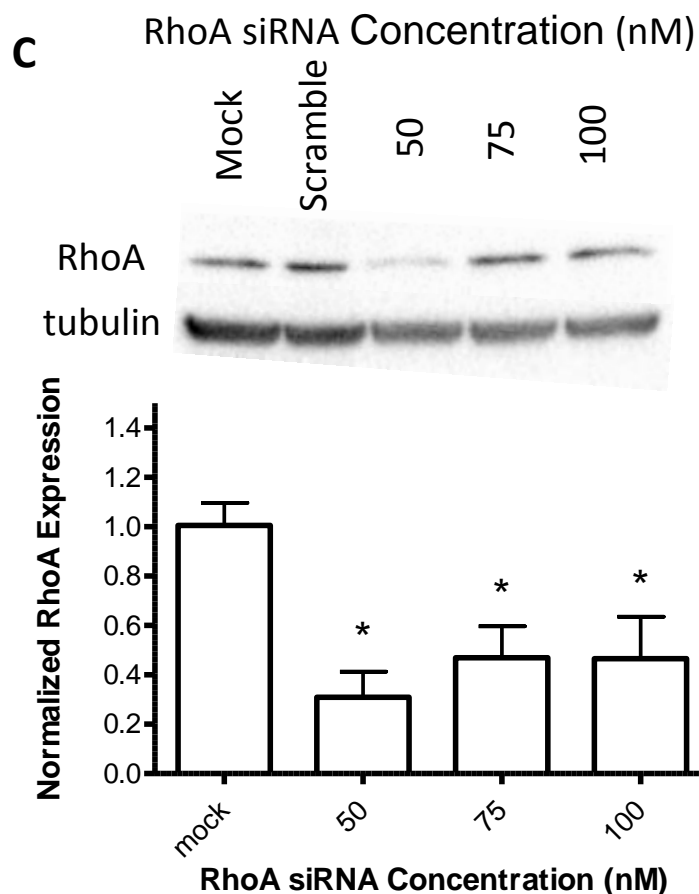


Figure 17. siRNA knockdown of RhoA decreases internalization of APP to lysosomes. A) N2A cells transfected with HA- β APP-CFP, Lamp1-chFP (red), a RhoA siRNA (s119551), and Stealth RNAi siRNA Negative Control Med GC-AlexaFluor 647 for 48 hours. Cells were then differentiated for a day and then immediately surface-labelled with Zenon-488 anti-HA antibodies (green) for 30 minutes, then incubated for 15 minutes at 37 °C. After 15 minutes of incubation at 37 °C, colocalization between HA- β APP and LAMP1 was observed. B) Quantification of colocalization analysis between HA- β APP and Lamp1 channels with standard error for 15 minute incubations at 37 °C for mock transfection, negative control, and RhoA siRNA. C) Western blot image and quantification with a mock, negative control, or 50 nM, 75 nM, or 100 nM concentrations of RhoA siRNA. Data retrieved from 3 replicates for Western Blots and 3 replicates for internalization, significance is denoted by * ($p < 0.05$). Scale bar = 5 μ m.

and progression of Alzheimer's (Iijima *et al.* 2008; Wiltfang *et al.* 2007). A Human A β 42 ELISA kit (Invitrogen) was used to analyze cell culture media after pharmacological inhibition. N2A cells were transfected with HA- β APP-CFP and then differentiated. After differentiation cells were incubated with a 0.1 % DMSO vehicle control, or inhibitors at 10 μ M for Rac1 (EHT 1864), Cdc42 (ML 141), or ROCKII (SR 3677) for 48h. This longer incubation time compared to the A β 40 experiments was used since cells produce so little A β 42 as compared to A β 40. After incubation, cell media was extracted and analyzed. Experiments were performed with 3 replicates, and data shown represents the mean \pm SEM normalized to the vehicle control, analyzed using a one-tailed t-test.

Production of A β 42 after inhibition of Rac1 (56.5 % \pm 9.3), Cdc42 (48.6 % \pm 8.0), and ROCKII (41.4 % \pm 5.8) were all significantly lower ($p < 0.05$) than control (109.6% \pm 4.8) (Figure 19). These results show that not only could pharmacological inhibition reduce A β 40 production, but also A β 42 production as well. Taking our results a step further, we divided the mean of the normalized A β 42 from the mean of the normalized A β 40, for each treatment, to examine the ratio of A β 42 to A β 40 production. The A β 42/A β 40 ratio after inhibition of Rac1, Cdc42, and ROCKII remained unchanged compared to control (essentially 1.0).

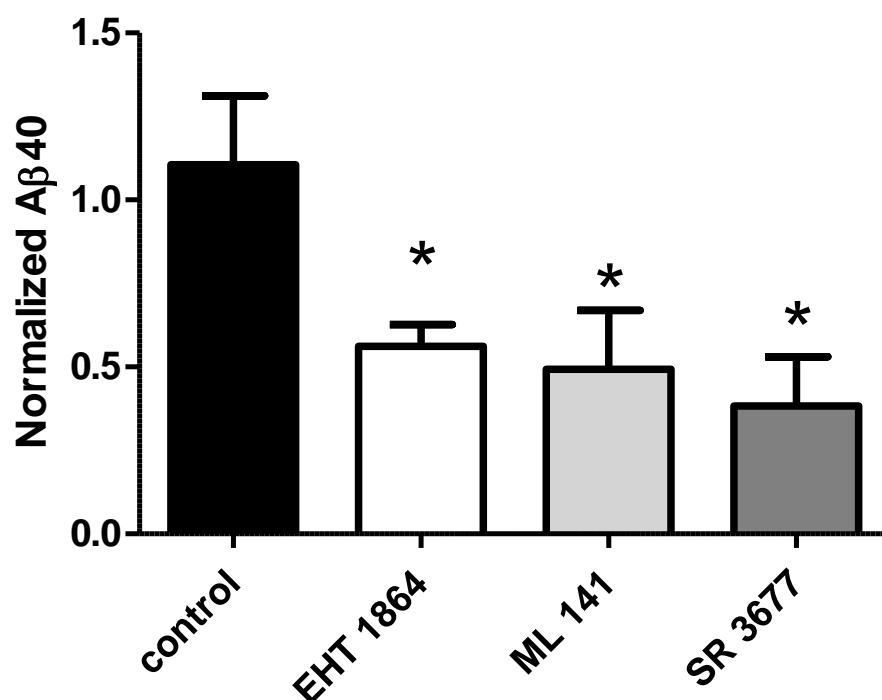


Figure 18. Aβ40 production is reduced after inhibition of Rac1, Cdc42, and ROCKII. A) N2A cells transfected with HA-βAPP-CFP for 24h then differentiated for 24h. Differentiation medium was then replaced with fresh media containing 0.1% DMSO, EHT1864 (Rac1 inhibitor), ML141 (Cdc42 inhibitor), or SR3677 (ROCKII inhibitor) for 24 hours. Immediately afterwards media samples were frozen and later analyzed using an Invitrogen Aβ40 ELISA according to manufacturer instructions. Data retrieved from 3 replicates, significance is denoted by * ($p < 0.05$).

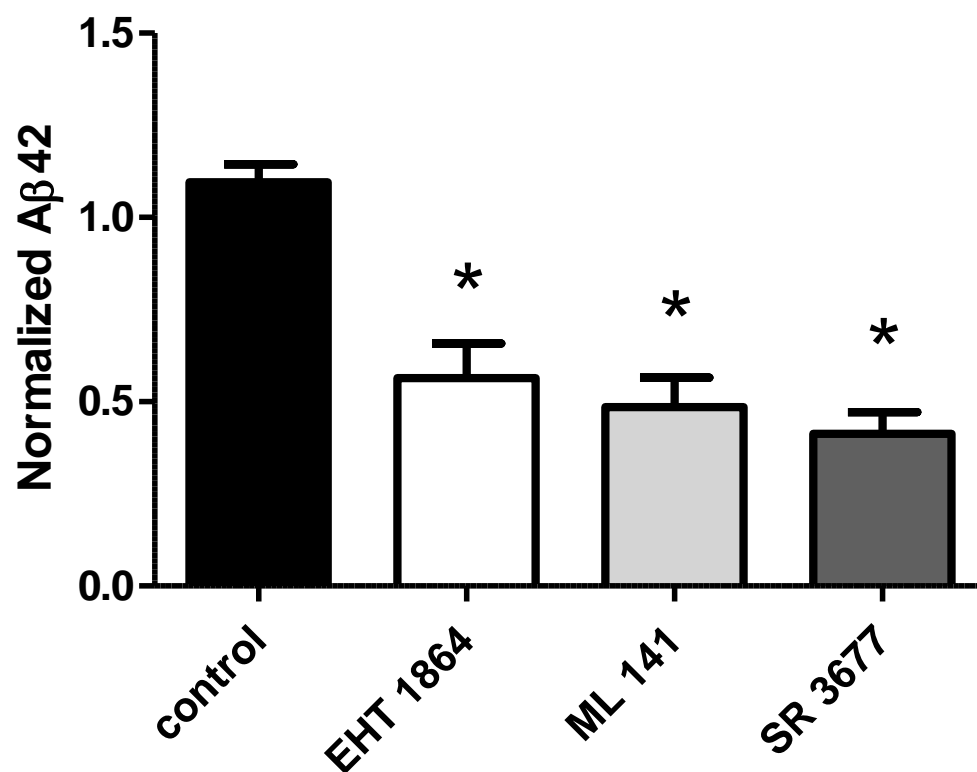


Figure 19. Aβ42 production is reduced after inhibition of Rac1, Cdc42, and ROCKII. A) N2A cells transfected with HA-βAPP-CFP for 24h then differentiated for 24h. Differentiation media was then replaced with fresh medium containing 0.1% DMSO, EHT1864 (Rac1 inhibitor), ML141 (Cdc42 inhibitor), or SR3677 (ROCKII inhibitor) for 24 hours. Immediately afterwards media samples were frozen and later analyzed using an Invitrogen Aβ42 ELISA according to manufacturer instructions. Data retrieved from 3 replicates, significance is denoted by * ($p < 0.05$).

Chapter 4: Discussion

Our laboratory has previously indicated that lysosomes may be significantly involved in the processing of APP (Pasternak *et al.* 2003), and that this process occurs through a rapid internalization of APP from the cell surface (Lorenzen *et al.* 2010). Furthermore, this process is mediated by the Arf6-GTPase, and may work through macropinocytosis (Tang *et al.* 2015). The purpose of this study was to identify possible effectors downstream of Arf6 to explore the pathway through which this APP trafficking mechanism occurs and also to see if inhibition or knockdown of these effectors could produce a molecular result visible through microscopy, which could carry through to changes in A β production. Through the use of fluorescently-labeled cell surface APP and compartment markers we were able to observe the trafficking of APP into both lysosomes and endosomes, following both our novel pathway and the classical pathway for APP transport in SN56 and N2A cells.

Using transfection of dominant negative and constitutively active mutants for Rac1, Cdc42, and RhoA we were unable to show any significant changes in APP transport to lysosomes. It is not clear why these constructs did not work in these cell lines. It is possible that the amount of mutant protein transfected into our cell lines was not enough to offset the normal function of endogenously produced Rac1, Cdc42, or RhoA. We verified that these constructs were transfected by imaging (they all had fluorescent tags), however the intensity of the fluorescent channel delineating the mutant proteins is not necessarily indicative of a noticeable effect. Additionally, though similar constructs have been shown to function in other cell types, it is possible that our mutant proteins are non-functional in N2A cells, for example if they do not interact properly with their required interaction partners in neuronal cells. Nevertheless, we showed that pharmacological inhibition of Rac1, Cdc42, and ROCKII could significantly reduce the amount of APP being transported to lysosomes. Furthermore, this reduced transport translated into significantly reduced levels of both A β 40 and A β 42 production, reinforcing a connection

between our proposed pathway and the endpoint of APP processing. While we observed none of these differences in APP trafficking to lysosomes using dominant and constitutively active mutants we were able to show significant changes through the use of siRNA knockdowns. In SN56 cells a Rac1 siRNA was able to elicit a significant decrease in APP transport to lysosomes, similar to the decrease observed using inhibitors. In N2A cells the same Rac1 siRNA was not able to produce a knockdown, possibly due to differences in protein expression or differences in siRNA transfection efficiency, however both Cdc42 siRNA and RhoA siRNAs were able to exhibit a decrease in APP transport to lysosomes, similar to their inhibitor counterparts.

4.1 Internalization of APP from the cell surface can be regulated through Rac1, Cdc42, and RhoA

Based upon a previous study in our lab that implicated Arf6 in the regulation of APP transport to lysosomes by macropinocytosis, a number of small GTPases were chosen for study. These GTPases have all been previously thought to be part of or necessary for macropinocytosis to occur and have all been suggested to interact downstream of Arf6 in the literature, except for Cdc42. Based upon the internalization experiments performed to observe APP transport, we observed results with Rac1, Cdc42 and RhoA that were highly similar to those that found previously with Arf6 (Tang *et al.* 2015).

Of the three Rho GTPases above, Rac1-GTPase is most strongly tied to macropinosome formation. Rac1 is essential for membrane ruffling, and eventually macropinosome formation, in a large number of cell types including macrophages and epithelial cells, requiring both activation and then deactivation of the GTPase for proper function (Fujii *et al.* 2013). Furthermore, it has been shown that ARNO, a guanine nucleotide exchange factor for Arf GTPases, activity leads to the activation of endogenous Arf6, and this activation leads to increased Rac1 activation. Perhaps more importantly, it was recently shown that an adaptor protein that binds APP, FE65, stimulates both Arf6 activation as well as Rac1 activation (Cheung *et al.* 2014). In this study Chinese hamster ovary (CHO)

cells were transfected to overexpress FE65 or transfected with an siRNA for FE65 knockdown, showing an increase or decrease respectively in Rac1 activity. This study also demonstrated that cells overexpressing FE65, but with Arf6 knocked down, were unable to activate Rac1, showing that Rac1 is downstream of Arf6 which is ultimately regulated by FE65. Taken together with the data in our study, this strongly suggests that APP may bind FE65, stimulating Arf6 activity, and further induce Rac1-mediated membrane ruffling for the endocytosis of APP. Our lab has also observed that mutation of tyrosine at the FE65 binding site was able to inhibit macropinocytosis, further implicating FE65 as a major player upstream of both Rac1 and Arf6 in the macropinocytosis of APP (unpublished results).

Cdc42, like Rac1, has been heavily implicated in the formation of macropinosomes, especially regarding its effects on actin polymerization and reorganization. Cdc42 has been shown to bind neural-Wiskott-Aldrich syndrome protein (N-WASP), which binds and then activates the Arp 2/3 complex (Matas *et al.* 2004; D'Souza-Schorey *et al.* 2006). This results in polymerization of an actin filament, forcing the plasma membrane out into a membrane ruffle and eventually macropinosome formation (Kerr *et al.* 2009). Furthermore, Cdc42-mediated N-WASP function requires binding of the Abi1, an essential component of the WAVE protein complex that is activated by Rac (Innocenti *et al.* 2005). It has also been shown that Cdc42 is likely downstream of Arf6 and that Arf6 activity may regulate Cdc42 activation and its effects on membrane dynamics and cell migration (Osmani *et al.* 2010; Jayaram *et al.* 2011). Taken together with our current data, the idea that Cdc42 acts downstream of Arf6 in our pathway for internalization of APP is reinforced. Additionally, the Cdc42-activated actin polymerization needed for macropinosome formation seems to be regulated by Rac1 helping to elucidate what role Rac1 may have in our suggested pathway.

RhoA has been shown to be an essential regulator of neuronal morphology, often with effects opposing Rac1. Often Rho upregulates apoptotic pathways, whereas Rac upregulates pathways for growth (Stankiewicz *et al.* 2014), requiring a balance between the two to maintain neuronal health, especially in dendritic growth (Newey *et al.* 2005). While not many studies have observed the effects of RhoA or any of the other Rho

subfamily GTPases a few have shown the presence of RhoA activity at the ruffles of membranes and in macropinosomes (Zawistowski *et al.* 2013; Pertz *et al.* 2006). Furthermore, RhoA localizes to the cell membrane unlike other Rho subfamily GTPases (Stankiewicz *et al.* 2014). While our data shows that knockdown or inhibition of RhoA has similar effects to Rac1 and Cdc42, it is possible that RhoA may be required for macropinocytosis to complete. As discussed above, Rac1 activation and then subsequent deactivation is required for macropinosome closure and maturation (Fujii *et al.* 2013). Similarly, RhoA activation and deactivation may be required for full macropinosome closure and maturation as well in a manner opposite to that of Rac1. This is supported through a study that shows that Arf6 activates Rac1 and Arf6 in opposite manners to regulate spine formation in neurons (Kim *et al.* 2015) and another study showing that ROCK2, a downstream effector of RhoA, attenuates Rac1 activity at the leading edge of lamellipodia and membrane ruffles (Newell-Litwa *et al.* 2015). In the same study, ROCK2 was also shown to activate cofilin, a protein that mediates actin depolymerization.

4.2 Building a pathway downstream of Arf6

Altogether the three Rho GTPases above appear to all be downstream of Arf6 and necessary for the regulation of actin dynamics and macropinosome formation. A proposed pathway visualizing the placement of just Arf6, Rac1, Cdc42, and RhoA (Figure 20) would place APP's binding to adaptor FE65 at the top, which is then able to bind to Arf6 (Cheung *et al.* 2015). Arf6 would then be able to activate Rac1 through some currently unknown mechanism, as well as possibly being able to activate Cdc42. Rac1, through the WAVE protein complex would be able to bind and activate the Arp 2/3 complex and promote membrane ruffling through actin polymerization (Innocenti *et al.* 2005). Rac1, through binding the WAVE complex would allow the Abi1 protein complex to activate N-WASP, allowing Cdc42 to bind to N-WASP. The binding of Cdc42 and N-WASP would also activate the Arp 2/3 complex and further promote actin polymerization and membrane ruffling. As the macropinosome forms and matures, Rac1 and Cdc42

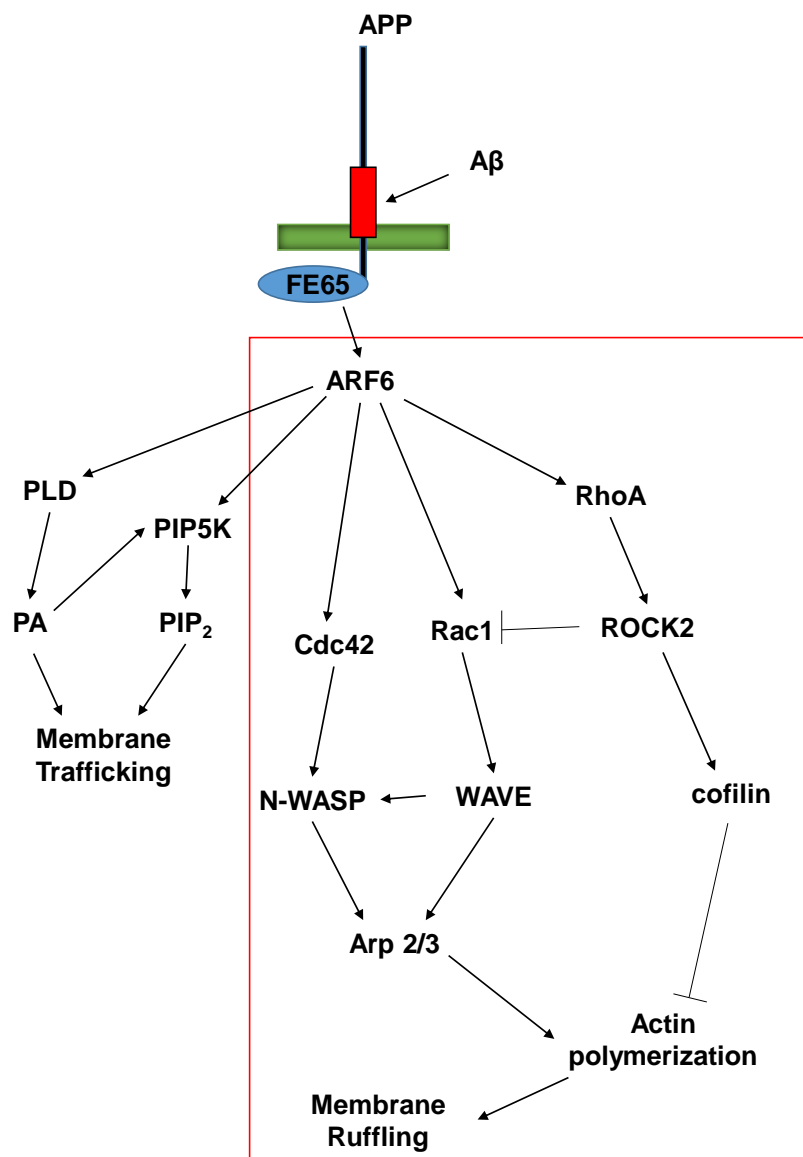


Figure 20. Proposed initial model for Arf6-mediated macropinocytosis of APP. Area in red represents the portion of this pathway focused on in this study. APP binding to FE65 activates Arf6, upregulating Cdc42, Rac1, and RhoA. Cdc42 and Rac1 activate the N-WASP and WAVE complexes, respectively. The WAVE complex is required to activate N-WASP. Both complexes bind and activate the Arp2/3 complex and initiate actin polymerization and membrane ruffling, leading to formation of a macropinosome. RhoA is activated at a delayed time, upregulating ROCK2 which attenuates Rac1 and activates cofilin. Cofilin and the attenuation of Rac1 lead to actin depolymerization and the eventual maturation and fission of the macropinosome from the membrane.

would need to be downregulated, while Arf6 upregulates RhoA simultaneously. RhoA would upregulate ROCKII, attenuating Rac1 and activating cofilin allowing the macropinosome to further mature and undergo fission from the cell membrane (Newell-Litwa *et al.* 2015).

4.3 Inhibition of APP Trafficking to Lysosomes Decreases in Production of A β 40 and A β 42

After observing significant decreases in the trafficking of APP to lysosomes with inhibition and knockdown of Rac1, Cdc42, and RhoA we wanted to know if these results would translate into observable differences in A β production. Of the two common A β peptides, A β 42 is considered to be more toxic (Iijima 2008; Kumar-Singh 2006). However, it has been suggested that not only is the absolute concentration of A β 42 important but also the ratio of A β 42 to A β 40 as well (Yin 2007). Furthermore, it has been shown previously that A β 40 may have a neuroprotective effect against A β 42 (Kumar-Singh 2006; Murray 2009). These studies suggest that A β 40 may actually inhibit A β 42 oligomerization or upregulate sequestration of A β 42 leading to increased clearance.

After analysis of our cells incubated with Rac1, Cdc42, and ROCK2 inhibitors it was revealed that inhibition of these three proteins was able to reduce A β 40 and A β 42 production. Furthermore, production was decreased by the same amount for both peptides. First, this shows that changes in APP transport are able to translate show a measured decrease in A β production. Although the A β 42/A β 40 ratio did not change significantly, the absolute concentration of A β 42 in the medium was still decreased, supporting the hypothesis that A β can be produced in the lysosome. Secondly, the role of Rac1, Cdc42, and ROCK2 is further reinforced in our novel pathway, as their inhibition was able to demonstrate a result similar to Arf6 in one of our previous studies (Tang *et al.* 2015). Inhibition of Rac1 showing a decrease A β production has been observed previously using EHT 1864, with similar results for A β 42 and A β 40 production (Désiré *et al.* 2005). Similarly, A β production has been previously shown to be decreased with

ROCK2 inhibition with a similar result (Herskowitz *et al.* 2013). While both these studies were the source of the incubation times and concentrations that we used, neither of these studies identified a mechanism for reduction of A β production. Our results extend the observations that these inhibitors reduce APP trafficking by macropinocytosis to lysosomes and provide the mechanism for the observed A β reduction, as well as providing support for the future use of these inhibitors in AD therapy. However, the previous study from Herskowitz *et al.* (2013) also visualized APP after incubation of the SR 3677 inhibitor. While we observed a decrease in APP transport to lysosomes that resulted in decreased A β production, their group observed an increase in BACE1 and APP to lysosomes after 6 h incubation. However, their study simply looked at all APP at the time of fixation. Our study observed only the movement cell surface-labelled APP into lysosomes after incubation; therefore, our study visualizes the trafficking of APP, whereas the study by Herskowitz *et al.* is really only looking at APP localization rather than trafficking. Furthermore, it is stated by Désiré *et al.* that the increased colocalization of BACE1 and APP with lysosomes was likely what lead to the decrease in A β production, but the mechanism through which this might occur is unclear. It is also possible that the concentration they used for ROCK2 inhibition was too high resulting in off-target effects. The concentration used was 50 μ M, a 5-fold increase higher than the concentration used in ours, and SR 3677 is able to inhibit ROCK1 as well at high enough concentrations, with an IC₅₀ 10-fold higher than that of ROCK2.

4.4 Current and Future Studies for Alzheimer's Disease Therapy

Much research is based around the Amyloid Cascade Hypothesis, with the overall goal of reducing A β production. Currently, many studies seek to inhibit the pathway directly through regulation of each step of the APP processing pathway. Some studies look at γ -secretase to inhibit the final γ -cleavage that produces A β 42 (Yin *et al.* 2007; Ling *et al.* 2015). However, PS1 has been shown to be required for proper neuronal development, so

the end results of inhibition of the γ -secretase are questionable (Shen *et al.* 1997). Other studies look at BACE1 inhibition, in order to stop the initial cleavage (Strömberg *et al.* 2015). Similar to studies looking to regulate γ -cleavage, studies looking to inhibit BACE1 may run into difficulty as BACE knockout mice exhibit significant neurological changes and BACE1 is involved in many functions such as axon growth, synapse function, and muscle spindle maintenance (Vassar *et al.* 2014). However, there are a number of BACE1 inhibitors currently in different phases of clinical trials (Vassar 2014). Another strategy for treatment of AD which has fallen into disfavour is the use of compounds meant to bind A β . This treatment was based upon the idea that aggregation of A β was what leads to cytotoxicity and neuronal death, however so far none have shown any positive results (Golde *et al.* 2011). As a result of these difficulties and previous failures, a promising avenue for research is soluble A β fragments referred to as Amyloid Derived Diffusible Ligands (ADDLs), which have shown to be extremely toxic to neurons as well as correlating with synaptic loss, which is one of the most important factors in the progression of Alzheimer's (Tam *et al.* 2012). Additionally, Soluble A β levels have been shown to correlate better with cognitive dysfunction much more closely than the appearance of plaques (Tomic *et al.* 2009).

Our findings provide a new method to regulate A β production by reducing APP transport to lysosomes, as well as outlining a number of new proteins to study further for therapy in Alzheimer's disease. While Rac1, RhoA, and Cdc42 have been strongly shown to be necessary for this the functioning of this pathway we still have little understanding how everything fits together. Although an intricate pathway has been proposed above (Figure 20) each step within the pathway needs to be verified if we are to truly understand the whole mechanism. Beginning with inhibition or knockdown of the FE65 adapter protein we should verify whether we are able to produce results similar to the internalization experiments shown in this study and our original Arf6 experiments. Although FE65 is what binds and activates Arf6, meaning it is upstream of Arf6 activation, it requires binding of the original APP molecule to FE65 in order to initiate the pathway cascade. If we are able to inhibit APP transport to lysosomes and therefore A β production at this first binding step, then we would be able to very specifically inhibit A β production without affecting normal cell physiology that requires the use of Arf6 or any of its downstream

effectors. However, it has also been shown previously in animal models that while single knockout models of FE65 family proteins appear normal, double knockout mice, for FE65 and FE65-like 1 (FE65L1), exhibit significant neurodevelopmental defects (McLoughlin *et al.* 2008). This points to some redundancy in the FE65 family of proteins, but still shows that they, and by extension APP processing, are extremely important to neuronal development. Therefore, it is also necessary to explore and validate the rest of the pathway to look for another therapeutic target. This can be done by run activation assays for phosphorylated GTPases after knockdown or inhibition of upstream effectors. For example, in order to verify that our pathway requires Arf6-activation in order for downstream RhoA upregulation, we can compare levels of phosphorylated RhoA with a control and an Arf6 knockdown. From this experiment we might expect a decreased level of phosphorylated RhoA with an Arf6 knockdown. Additionally, we could knockdown Arf6 for example, while transfecting a constitutively active mutant for Rac1 in order to see if we could rescue some of the observed attenuation of APP transport to lysosomes. This could help tease out which proteins in the pathway are really upstream or downstream from others, as it is entirely possible that the GTPases examined in this study work at the same time as and not downstream of Arf6. In addition, the functionality of the Rac1, Cdc42, and RhoA mutants should be examined following the negative results outlined above. An experiment that could be performed for mutant functionality could look at phalloidin conjugate stains for actin filaments after transfection with the dominant negative and constitutively active constructs to observe their effects on actin dynamics and ruffle formation. We must also further verify whether Rac1, Cdc42, and RhoA affect our pathway through regulation of macropinocytosis. This could also be determined through the staining of actin filaments with phalloidin conjugates to visualize effects of inhibition of formation of membrane ruffles (Boshans *et al.* 2000), or through the visualization of macropinosomal markers such as SNX5 (Kerr *et al.* 2006). However, markers of macropinosomes such as SNX5 also localize to endosomes as well making differentiation between the two compartments difficult (Merino-Trigo *et al.* 2004). Furthermore, we should examine whether our results regarding APP transport and A β production can translate into neurons and mice for future clinical applications. In particular, SR3677 has already been used previously in mice with stereotaxic injections

with a similar decrease in A β production (Herskowitz *et al.* 2013). Similarly, EHT1864 has been shown to demonstrate decreases in A β production in guinea pigs (Désiré *et al.* 2005). However, further application of these inhibitors such as for clinical studies are will need to be performed.

4.5 Conclusions

This study provides the foundation for further study of an FE65-mediated pathway that results in the endocytosis of APP to lysosomes. Our lab previously identified a novel and direct pathway for the transport of APP to lysosomes, a possible production site for A β . This was expanded further to identify Arf6 as a possible candidate for the initiation of the endocytosis of APP through a macropinosome-like pathway. This was reinforced through observation that an Arf6 knockdown also exhibited reduced A β production. Our current study further demonstrates that the Rho GTPases, Rac1, Cdc42, and RhoA, were able to demonstrate results comparable to those previously observed with Arf6. All three GTPases showed decrease trafficking of cell-surface labeled APP through pharmacological inhibition as well as small interfering RNA knockdown. These results are supported by previous studies that indicate that Rac1, Cdc42, and RhoA are all downstream effectors of Arf6. A possible model for this pathway is also proposed where FE65 may bind APP, leading to the activation of Arf6. Arf6 would then activate Rac1 and Cdc42, which promote membrane ruffling and macropinosome formation. RhoA would also be activated at a later time leading to actin depolymerization and macropinosome maturation. The macropinosome would then undergo fission from the cell membrane, and later fuse with a lysosome, transporting APP to its site of production. Additionally, using inhibitors for the above GTPases we were also able to observe significant decreases in both A β 40 and A β 42 production. These results have been observed previously, however other studies have never connected observable changes in APP transport from the cell surface to lysosomes with these proteins to changes in A β production as we have here. Further work should be done to further examine each step of this pathway starting from APP binding to FE65, and then moving down the pathway

looking for a target for therapy that is specific enough to inhibit this pathway while leaving other important pathways intact. Hopefully these results will be able to further our understanding of APP trafficking in Alzheimer's disease and promote the discovery of a cure in the future for this disease.

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